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(71) Applicant *(for all designated States except US)*: NEUREX CORPORATION [US/US]; 3760 Haven Avenue, Menlo Park, CA 94025-1012 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : JUSTICE, Alan [US/US]; 1280A Poplar Avenue, Sunnyvale, CA 94086 (US). SINGH, Tejinder [US/US]; 1537 Louisa Court, Palo Alto, CA 94303 (US). GOHIL, Kishor, Chandra [GB/US]; 6217 Plymouth Avenue, Richmond, CA 94805 (US). VALENTINO, Karen, L. [US/US]; Two Elm Street, No. 202, San Carlos, CA 94070 (US). MILJANICH, George, P. [US/US]; 3623 Glenwood Avenue, Redwood City, CA 94062 (US).

(74) Agent: STRATFORD, Carol, A.; Law Offices of Peter J. Dehlinger, P.O. Box 60850, Palo Alto, CA 94306 (US).

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(54) Title: METHODS OF PRODUCING ANALGESIA AND ENHANCING OPIATE ANALGESIA

(57) Abstract

Methods of producing analgesia and enhancing opiate analgesia are disclosed. The methods include administering a TVIA (SNX-185) or MVIIA (SNX-111) omega-conopeptide, or derivative thereof which is characterized by its ability to (a) inhibit voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the peptide's ability to inhibit electrically stimulated contraction of the guinea pig ileum, and (b) bind to omega conopeptide MVIIA binding sites present in neuronal tissue. Also disclosed is a novel omega conotoxin peptide derivative effective in producing analgesia.

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METHODS OF PRODUCING ANALGESIA AND ENHANCING OPIATE ANALGESIA

1. Field of the Invention

The present invention relates to methods of producing analgesia and of enhancing opiate analgesia, particularly in the treatment of pain and neuropathic pain.

2. References

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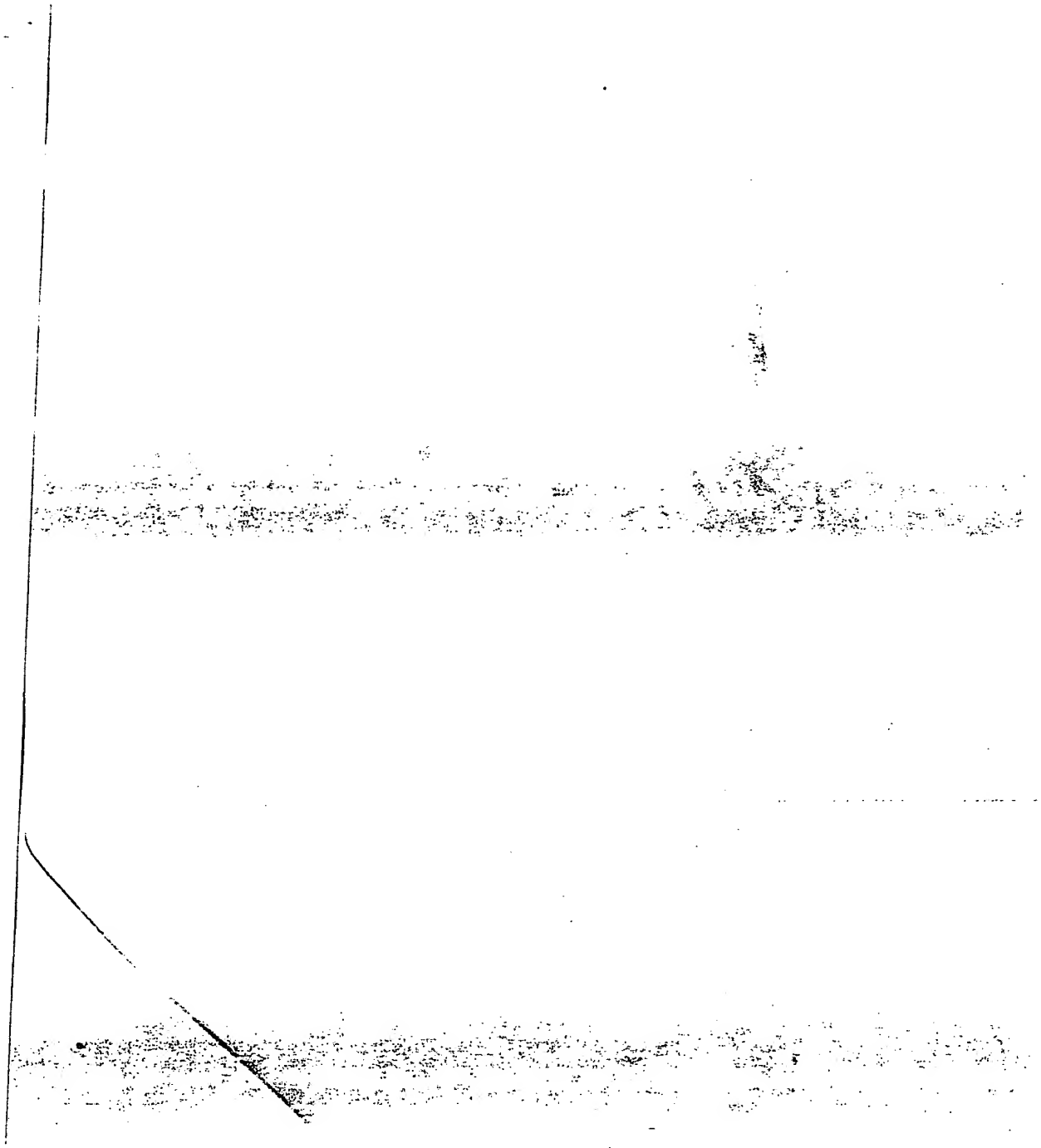

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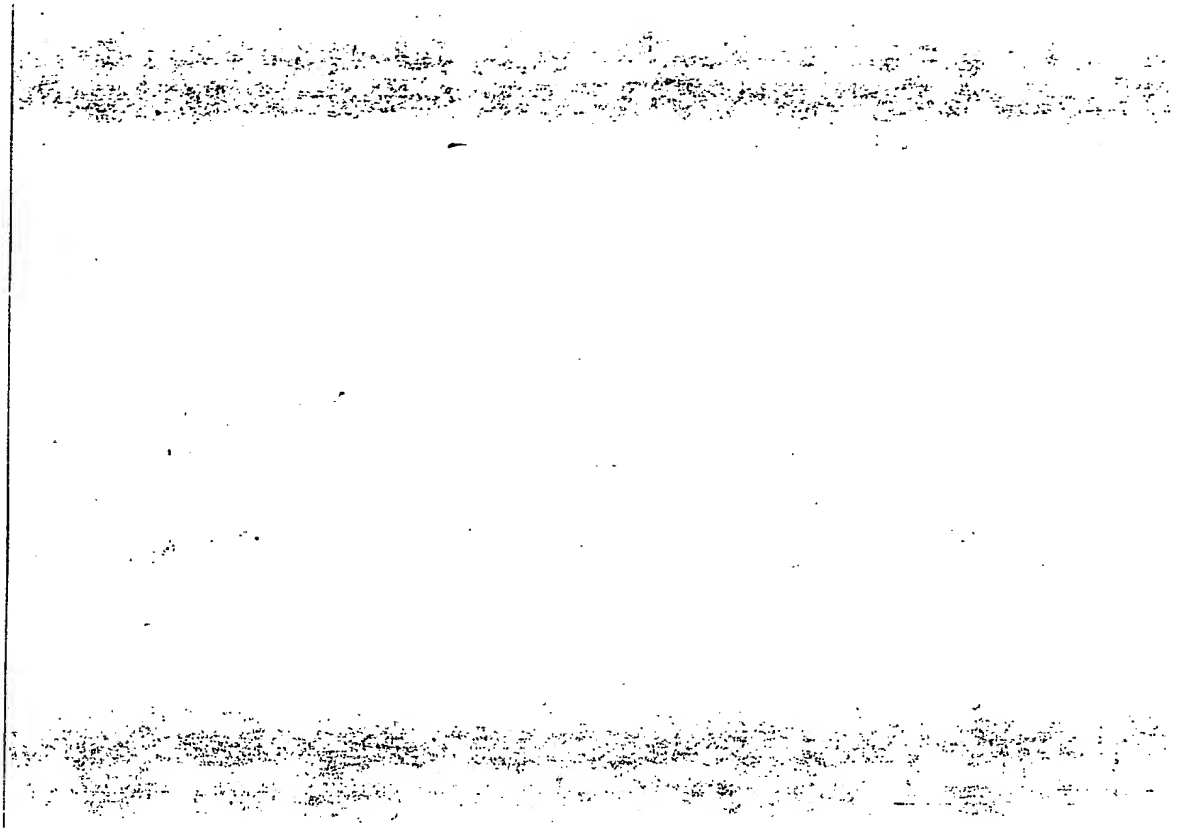

ic pain are widely sought after. In addition, unds which serve as either a replacement for or as junct to opioid treatment in order to decrease the e of analgesic compound required, have utility in reatment of pain, particularly pain of the c, intractable type.

lthough calcium blocking agents, including a of L-type calcium channel antagonists, have been as adjunct therapy to morphine analgesia, ve results are attributed to direct effects on availability, since calcium itself is known to te the analgesic effects of certain opioid ds (Ben-Sreti). EGTA, a calcium chelating s effective in increasing the analgesic effects ids. However, results from tests of calcium sts as adjunct therapy to opioids have been ctory; some L-type calcium channel antagonists en shown to increase the effects of opioids, hers of these compounds have been shown to opioid effects (Contreras).

Patent No. 5,051,403 describes the use of opeptides having defined binding/inhibitory s in the treatment of ischemia-related damage. In the present invention, it has been omega-conopeptides having related inhibitory ing activities produce analgesia. In these compounds also enhance the analgesic sub-threshold levels of opioid compounds in subjects.

y of the Invention

vention includes, in one aspect, a method of the analgesic effect produced by ion of opiates to a mammalian subject. In pect, the invention includes a method of analgesia in a mammalian subject. The

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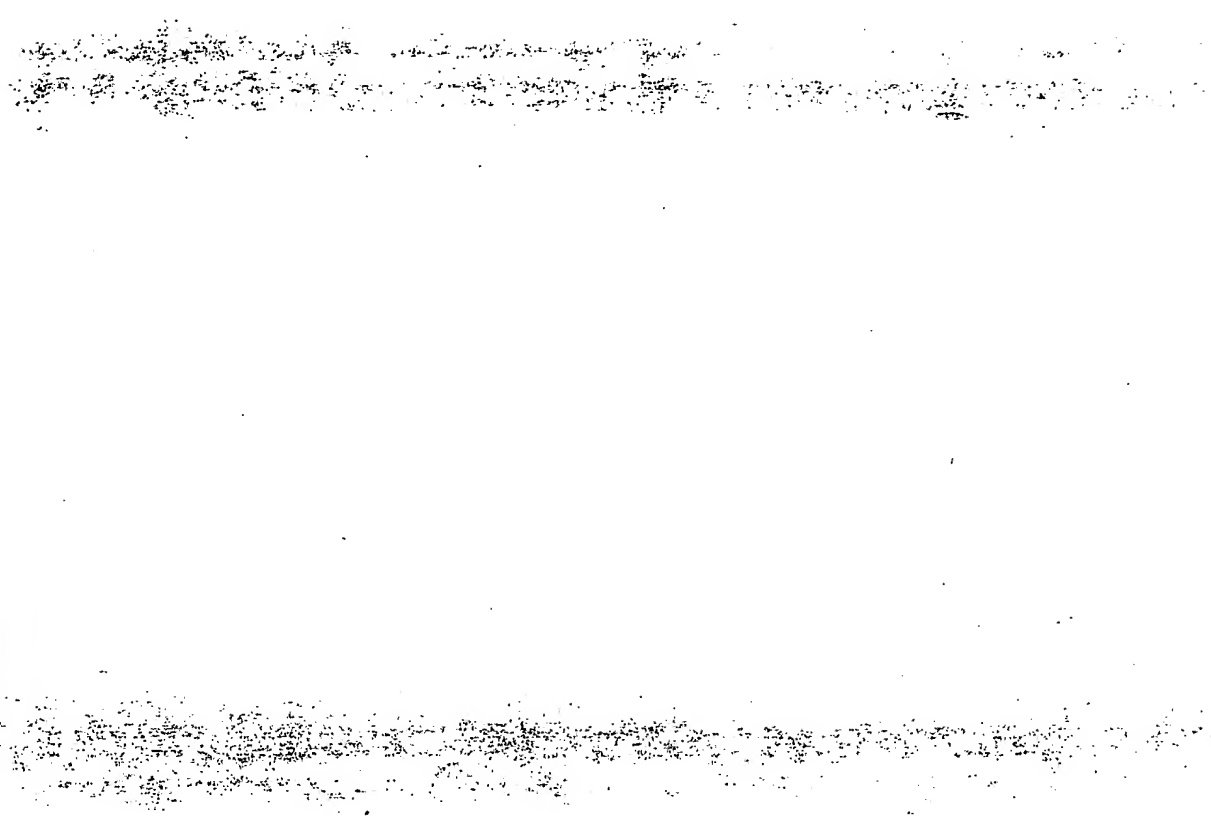

Figure 2 shows several analog omega-conopeptides SNX-190 (SEQ ID NO: 09), SNX-191 (SEQ ID NO: 10), SNX-193 (SEQ ID NO: 11), SNX-194 (SEQ ID NO: 12), SNX-195 (SEQ ID NO: 13), SNX-196 (SEQ ID NO: 14), SNX-197 (SEQ ID NO: 15), SNX-198 (SEQ ID NO: 16), SNX-200 (SEQ ID NO: 17), SNX-201 (SEQ ID NO: 18), SNX-202 (SEQ ID NO: 19), SNX-207 (SEQ ID NO: 20), SNX-236 (SEQ ID NO: 30) and their relationships to SNX-111 (SEQ ID NO: 01), SNX-185 (SEQ ID NO: 07) or SNX-183 (SEQ ID NO: 08);

Figures 3A-3D show voltage-gated calcium current traces induced by a voltage step from -100 or -80 mV to -20 mV in untreated N1E-115 neuroblastoma cells (3A) and in neuroblastoma cells exposed to increasing concentrations of OCT MVIIA (SNX-111) (3B-3D);

Figure 4 plots the percent inhibition of peak inward calcium currents in neuroblastoma cells as a function of OCT MVIIA (SNX-111) (solid triangles) and OCT GVIA (SNX-124)(solid circles);

Figure 5A shows voltage-gated calcium current traces induced by a voltage step from -70 to -20 mV in human neuroblastoma cells (IMR-32) in the absence (lower trace) and presence (upper tracing) of 150 nM SNX-111;

Figures 5B and 5C show plots of absolute values of peak inward current measured every 15 seconds in IMR-32 cells, elicited by pulses from -70 to 0 or -10 mV, versus time, where addition of compounds SNX-111 (5B) or SNX-111, SNX-183 (5C), and cadmium to the bathing medium are indicated by hatch marks just above the ordinate;

Figures 6A and 6B are a binding curve showing the amount of radioiodinated OCT MVIIA (SNX-111) bound to rat synaptosomal membranes, as a function of OCT MVIIA (SNX-111) concentration (6A), and the same data plotted as a Scatchard plot (6B);

Figures 7 shows reversibility of equilibrium binding of radioiodinated SNX-111 and SNX-183, and irreversibility of binding of radioiodinated SNX-124 to rat brain synaptosomal membranes;

Figures 8A and 8B show computer-fit competitive binding curves for omega-conopeptide binding to the OCT MVIIA (SNX-111) binding site in rat brain synaptosomes;

Figure 9A shows computer-fit competitive binding curves for omega-conopeptide binding to the OCT SVIB (SNX-183) binding site in rat brain synaptosomes, and Figure 9B shows binding site competitive binding curves for binding to OCT MVIIC (SNX-230);

Figure 10 (A and B) shows SDS-PAGE autoradiograms of rat synaptosomal membranes having covalently bound radioiodinated OCT MVIIA (SNX-111)(A) or covalently bound radioiodinated OCT SVIB (SNX-183)(B) added to the membranes in the presence (lanes c and f) or absence (lanes a,b and d,e) of non-radiolabeled OCT, where lanes a and d are control preparations in which no cross-linking agent was added;

Figure 11 shows plots of competition by unlabeled SNX-111 and SNX-183 for binding of [^{125}I]-SNX-111 (11A) and [^{125}I]-SNX-183 (11B) to the 210 kilodalton band of polypeptides present in rat synaptosomal membranes;

Figure 12 shows autoradiograms of the distributions of [^{125}I]-SNX-111 (A,B,C,D) and [^{125}I]-SNX-183 (E,F,G,H) binding to coronal rat brain rostral (A,C,E,G) and caudal (B,D,F,H) sections labeled in the absence of excess nonradioactive SNX-III (A,B) or SNX-183 (E,F) or in the presence of excess non-radioactive SNX-111 (C,D) or SNX-183 (G,H), in which "CA" indicates the CA_3 region of the hippocampus and "SN" indicates the substantia nigra;

Figure 13A shows the inhibition of [^{3}H]norepinephrine release from rat hippocampal slices

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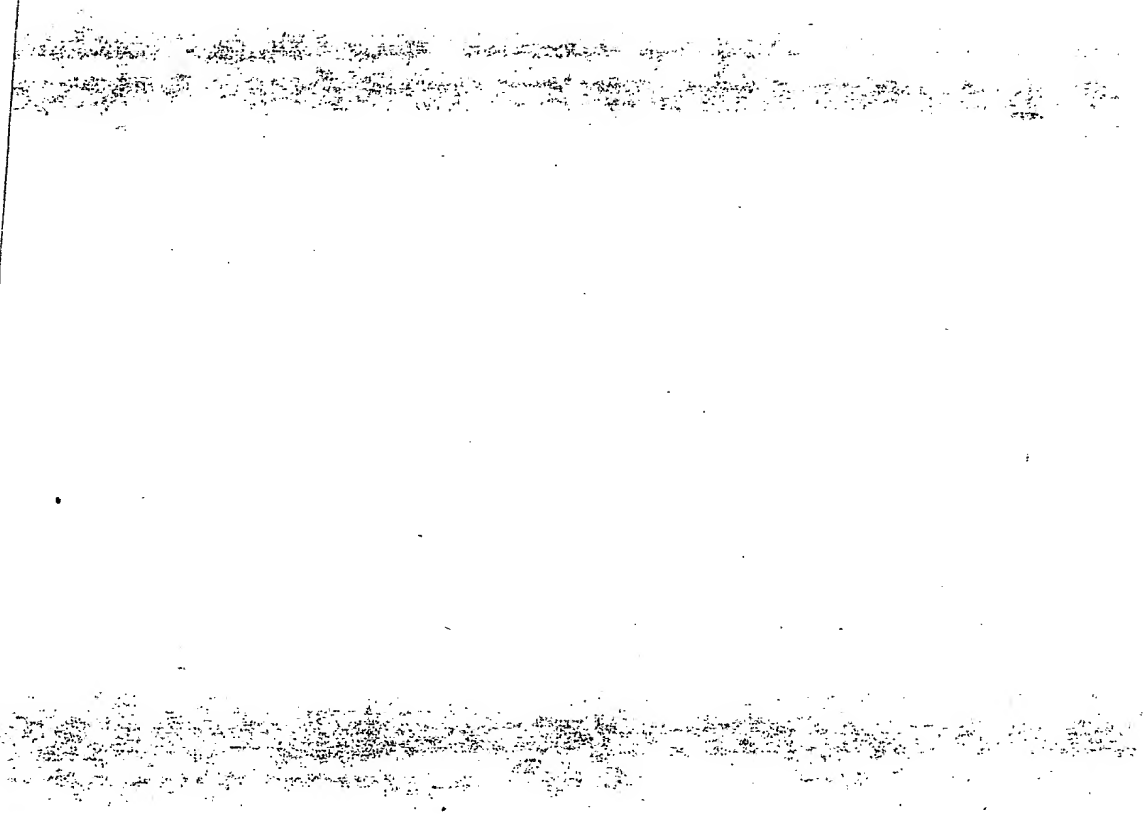

omega-conopeptides are shown in Figure 1, where SNX-231 is an alternative form of MVIIC/SNX-230. Conventional letter initials are used for the amino acid residues, and X represents 4-hydroxyproline, also abbreviated 4Hyp. All of the peptides shown in the figure are amidated at their C-termini.

The peptides shown in Figure 1 are identified by names which are commonly associated with either the naturally occurring peptide (single letter followed by a Roman numeral followed by a single letter), and by a synthetic designation (SNX-plus numeral). Either or both of these designations will be used interchangeably throughout the specification. For example, the peptide whose sequence is designated MVIIA/SNX-111 will be referred to herein as OCT MVIIA, or alternatively, SNX-111, the latter to signify that the compound is synthetic in origin. Synthetic and naturally occurring peptides having the same sequence behave substantially identically in the assays and methods of treatment of the invention. The OCT MVIIA (SNX-111) and OCT GVIA (SNX-124) peptides also have the common names CmTx and CgTx, respectively. All of the omega-conopeptides have three disulfide linkages connecting cysteine residues 1 and 4, 2 and 5, and 3 and 6, as indicated for the MVIIA peptide in Figure 2. Figure 2 shows analogs or derivatives of natural OCT MVIIA, OCT TVIA, and OCT SVIB peptides which have been synthesized and tested in accordance with the invention. Standard single amino acid code letters are used in the figure; X=hydroxyproline; Nle=norleucine; NH_2 group at the C terminus indicates that the peptide is C-terminal amidated; G-OH indicates termination in an unmodified glycine residue.

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f Omega-conopeptides
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Electrophysiologically, these channels can be assified either as Low-voltage-activated (LVA) or gh-voltage-activated (HVA). HVA channels are rrently known to comprise at least three groups of annels, known as L-, N- and P-type channels)wycky, Sher). These channels can be distinguished ctrophysiologically as well as biochemically on the is of their pharmacology and ligand binding)perties. Thus, dihydropyridines, henylalkylamines and piperidines bind to the alpha, nit of the L-type calcium channel and block a)ortion of HVA calcium currents in neuronal tissue, h are termed L-type calcium currents.

Omega conotoxins also block a proportion of HVA ium currents in neuronal tissue, and, in the ence of a maximally inhibitory quantity of dropyridine compound, substantially inhibit the ining HVA currents in neuronal cells. These um currents are generally identified as N-type um currents. Based on their pharmacological tivity, such currents are also termed "omega" nts by some authors (Sher).

)mega conotoxins bind to a specific population of g sites, present mainly in neuronal tissue. opyridines and other L-type channel blockers do splace omega conotoxin binding, nor do omega xins displace binding of ligands to L-type channels. Unlike L-type calcium channels, N-)r omega channels are found predominantly, h not exclusively, in nervous tissue (Sher).

e suitable system for testing inhibition ge) of N-type or omega HVA neuronal calcium s is an isolated cell system, such as the mouse astoma cell line, strain N1E115 or the human

:oma cell line IMR32. Membrane currents are :ly measured with the whole cell configuration :ch clamp method, according to the procedure in Example 1. Briefly, a voltage clamp as performed in which the cell potential was om the holding potential of about -100 mV to :ials that ranged from -60 mV to +20 mV, and vas he.. at the holding potential for 5 :ween pulses.

3 shows a typical inward calcium current a voltage step from -80 mV to -20 mV in the OCT. In this, and most of the recordings :um (Ba) replaced calcium (Ca) as the :er through the calcium channels in order to e signal (McCleskey). According to the described in Example 1, an N1E115 :a cell was bathed in saline with sodium N-methyl-D-glucamine (NMDG), and 10 mM Ba mM Ca. These substitutions reduced the nt that would otherwise have contaminated current record, and increased the calcium what it would have been with only 2 mM Ca Potassium currents were blocked by onium (TEA) in the bath and cesium (Cs) in ution.

from Figure 3, curve A, the calcium ates quickly (within about 20 ms) and :th a time constant of 30 to 40 ms. The nt is measured by the amplitude of the current elicited by the depolarization a measured value of about -1200 pA. The e 3 (curve A) was also exposed to 1μM dihydropyridine, which is expected to lock L-type calcium channels in the

neuroblastoma cells, and no effect on the measured calcium current was observed. The calcium current observed is thus not dihydropyridine-sensitive.

The responses of voltage-gated calcium currents to increasing concentrations of OCTs MVIIA (SNX-111) and GVIA (SNX-124) are shown in Figure 4. The IC_{50} concentration, at which 50% inhibition of calcium current is produced, is determined from the voltage-gated current amplitudes, plotted as a function of omega-conopeptide concentration. The calculated IC_{50} is about 10 nM for GVIA and 100 nM for MVIIA, indicative of high inhibitory peptide activity. The IC_{50} concentration for these and omega-conopeptides SVIA (SNX-157) and SVIB (SNX-183) are given in Table 1 below.

Table 1

Inhibition of calcium currents in N1E-115 neuroblastoma cells

Compound	IC_{50}
GVIA (SNX-124)	10nM
MVIIA (SNX-111)	100nM
SVIB (SNX-183)	> 1μM
SVIA (SNX-157)	>20μM

Calcium currents were also measured in human neuroblastoma IMR32 cells, using techniques described above and in Example 1. Voltage-gated calcium currents were elicited by holding the cell(s) at -70 mV and administering a step-voltage to -10 mV. Current tracings from IMR-32 cells bathed in control medium (lower curve) and in medium containing 150 nM SNX-111 (upper curve) are shown in Figure 5A. The amplitude of the current is shown on the abscissa. The peak inward

current is shown as the difference between the resting potential shown at the far left side of the figure and the lowest point of the curve, just adjacent to the resting value. In this experiment attenuation of voltage-gated calcium current is apparent in the presence of SNX-111 (upper curve), as shown by the decreased amplitude of the peak inward current.

Figures 5B and 5C show cumulative data from many consecutive currents, elicited at 15 second intervals as described above, in IMR-32 cells. In these plots, peak inward current recorded from each stimulus is recorded sequentially as a single data point. In the experiment illustrated in Figure 5B, addition of SNX-111 to the bathing medium resulted in decreased peak inward currents; restoration of substantially normal calcium currents was achieved after washing of the compound from the cell chamber, shown on the right side of the figure. Figure 5C shows the effects of 150 nM SNX-111 and SNX-183 added sequentially to a single cell preparation. Both compounds resulted in attenuation of peak inward current; though recovery following SNX-183 exposure was not observed. Addition of cadmium (Cd) to the medium resulted in blockade of all remaining voltage-gated calcium currents in this preparation.

Test peptides which are inhibitory for neuronal cell calcium currents can be further tested in non-neuronal cells, to confirm that the peptide activity in blocking calcium currents is specific to neuronal cells. A variety of muscle cell types which are refractory to calcium-current inhibition by OCTs, such as vertebrate embryo heart and skeletal muscle cells, are suitable. Cell current measurements are made substantially as outlined above and detailed in Example 1. OCT MVIIA, for example, has been reported to block voltage-gated calcium channels in a variety of neuronal

including dorsal root ganglion (DRG) neurons skey). This blockage or inhibition of calcium currents has been reported to be neuron-c, since calcium current inhibition by the was not observed in cardiac, smooth, and l muscles.

cific, High Affinity Binding to OCT Receptors

ega-conopeptides have been shown, in accordance ie invention, to bind with high affinity to binding site(s) in neuronal cells. In nce with the selectivity of the compound, the affinity can be characterized either by the constant of the compound for the MVIIA (SNX-ding site, also referred to as "site 1" herein, binding constant of the compound for the SVIB) or the MVIIC (SNX-230) binding site, also to as "site 2" herein. Evidence for the e of at least two distinct OCT binding sites is ed below. In some cases, when specific binding site is preferred, it will be useful to rize omega-conopeptides according to the ratio binding constants measured for binding to -cell MVIIA (SNX-111) binding site 1 and SVIB or MVIIC (SNX-230) binding site 2.

ling to the OCT MVIIA binding site in neuronal n be demonstrated in a variety of cell types ptosomal cell fractions. One preferred mal fraction is a mammalian brain synaptosomal preparation, such as the rat brain synaptosome on described in Example 2. The binding of a compound for the MVIIA binding site is determined by competitive displacement of led OCT MVIIA (SNX-111) from the synaptosomal on, as follows.

The binding constant K_d of the MVIIA (SNX-111) peptide for the synaptosomal membranes is determined by a saturation binding method in which increasing quantities of radiolabeled peptide are added to the synaptosomal membranes, and the amount of labeled material bound at each concentration is determined (Example 3A). The plot of bound peptide as a function of concentration is then used to calculate a B_{max}, the concentration of binding sites on the synaptosomes, and K_d following standard methods. In particular, the K_d value is the calculated concentration of peptide needed to half saturate the synaptosomal specific binding sites. Figure 6A shows the specific binding of radiolabeled OCT MVIIA (SNX-111) to rat brain synaptosomes, plotted as a function of omega-conopeptide concentration, and Figure 6B, the same data in Scatchard plot form. From the slope of the Scatchard plot line, a K_d binding value of 10 pM is obtained. Similarly K_d's were determined for binding of radiolabelled SVIB (SNX-183) to binding sites in synaptosomal membranes.

Reversibility of binding is a characteristic of ligands which, under equilibrium conditions, freely associate with and dissociate from their respective binding sites. Reversibility of binding of a specific compound is demonstrated by the labelled compound's ability to be displaced by unlabelled compound, after equilibrium binding of the labelled compound has been achieved. For example, dissociability of binding of a labelled compound can be determined as detailed in Example 3B, where a synaptosomal preparation was incubated with labelled compound for a time period sufficient to produce a stable level of binding, then excess unlabelled compound was added to the preparation. The preparation was then assayed for

bound labelled compounds at various timepoints after addition of unlabelled compound.

If the labelled compound binds reversibly to the preparation, a reduction of labelled binding, to essentially non-specific binding levels, should be observed over time. Figure 7 shows a plot of the dissociation kinetics of labelled SNX-111, SNX-183 and SNX-124. In contrast to SNX-111 binding, labelled OCT GVIA (SNX-124) does not dissociate from synaptosomal membranes over the course of an hour and has a calculated $t_{1/2}$ of 19 hours. SNX-124 binding can therefore be said to be essentially irreversible, while SNX-111 and SNX-183 bind reversibly to their respective binding sites.

To determine the binding constant of a test compound for an OCT binding site, the test compound is added, at increasing concentrations, to the synaptosome preparation in the presence of a standard concentration of a radiolabeled OCT which exhibits reversible binding, such as OCT MVIIA (SNX-111). The synaptosomal material is then rapidly filtered, washed and assayed for bound radiolabel. The binding constant (K_i) of the test compound is determined from computer-fit competitive binding curves, such as shown in Figures 8A and 8B for MVIIA (SNX-111) peptide, to determine first the IC_{50} value of the compound, i.e., the concentration which gives 50% displacement of labeled MVIIA peptide. A K_i is determined according to standard methods from the K_d value of OCT MVIIA and the IC_{50} value of the compound, as detailed in Example 3. A relative potency value can also be calculated from this information Example 3). Like the K_i value, this value allows comparisons between assays performed under slightly differing conditions or at different times. Calculated IC_{50} values for a number of omega-conopeptides for

binding of OCT MVIIA (SNX-111) are given in Table 2. The compounds are arranged in order of increasing IC_{50} values.

Table 2
Competition of ^{125}I-MVIIA (SNX-111) Binding by OTC Peptides

	IC_{50} (nM)
SNX-200	.007
SNX-194	.008
SNX-195	.009
MVIIA (SNX-111)	.013
SNX-190	.021
SNX-200	.039
SNX-201	.046
SNX-202	.069
SNX-193	.070
MVIIC (SNX-230)	.32
MVIIB (SNX-159)	.101
GVIA (SNX-124)	.134
SNX-198	.160
SNX-191	.165
TVIA (SNX-185)	.228
SNX-196	.426
RVIA (SNX-182)	.893
SVIB (SNX-183)	1.5
GVIIA (SNX-178)	3.70
SNX-197	11.3
SVIA (SNX-157)	1460.

Similarly, IC_{50} and K_i values for compound binding to the SVIB (SNX-183) binding site can be calculated,

as above, by determining the K_d of labeled OCT SVIB (SNX-183) or OCT MVIIC (SNX-230) binding to a synaptosome preparation, then using competitive displacement of the labeled compound by the test compound, to determine the IC_{50} and K_i or relative potency values of the test compound. Figures 9A and 9B show computer-fit competitive binding curves for several omega-conopeptides whose binding to the SVIB (SNX-183) and MVIIC (SNX-230) binding sites was examined. From these curves, IC_{50} values were determined as above.

Table 3 lists the relative potencies for binding of various omega-conopeptides to the site 1 and site 2 binding sites, and shows the ratio of Ki values determined for binding of each compound to the sites.

Table 3

Selectivity of Conopeptides for Site 1 and Site 2

Compound	Ki (nM) for competition[a] with:		Selectivity[b] for:	
	[^{125}I]-SNX-111	[^{125}I]-SNX-230	site 1	site 2
SNX-111	0.002	150	75,000	: 1
[^{127}I]-SNX-111	0.002	320	39,400	: 1
SNX-183	0.43	6	14	: 1
[^{127}I]SNX-183	1.25	1.5	1.2	: 1
SNX-230	0.20	0.03	1	: 7
[^{127}I]-SNX-230 (SNX-260)	0.49	0.02	1	: 25

[a]Ki values were derived from analysis of competitive binding performed as described in Figure 1.

[b]Selectivity is expressed as the ratio of the Ki value determined for competition with [^{125}I]-SNX-230 binding divided by the Ki value for competition with [^{125}I]-SNX-111 binding.

the MVIIA and SVIB binding sites
ıbranes was examined by binding
MVIIA to synaptosomes, and
ide to the neuronal membranes, as
4. The labeled membranes were
odium dodecyl sulfate (SDS),
/acrylamide gel electrophoresis
by autoradiography for labeled
In one case, the membranes were
ed peptide in the presence of
MVIIA. A similar binding study
.abeled OCT SVIB.

two receptor sites identified by
are distinct was obtained from
studies in which [^{125}I]-SNX-111
chemically crosslinked to rat
embrane preparations and then
llowed by autoradiography (Fig.
.ifically labelled a protein of
indicated in the drawing.
ibited labeling of this protein
1 with an IC_{50} of 30 pM, in good
or site 1 determined by binding
ng of this 210 kDa protein band
o inhibited by SNX-183 but with
0pM). Similar experiments with
hat in addition to the expected
KDa, three additional bands at
ear to be specifically labeled

ıhibition of incorporation of
0kDa band by SNX-111 provides
e of two distinct polypeptides
ng to site 1 and site 2 (Fig.
[^{125}I]-SNX-183 from the 210kDa

polypeptide in a biphasic manner with IC_{50} values of 6 pM and 65 nM. At low concentrations, SNX-111 effectively competed against [^{125}I]-SNX-183 for binding to site 1, while the binding of [^{125}I]-SNX-183 to site 2 was competitively displaced by SNX-111 only at much higher concentrations.

Inhibition of incorporation of [^{125}I]-SNX-183 into the 210kDa band by SNX-183 is consistent with the ability of this compound to bind to both site 1 and site 2, but, as shown above, with much lower affinity than MVIIA (SNX-111) or TVIA (SNX-185) at site 1. The displacement curve displacement of MVIIA binding by SNX-183 is characteristically shallow, with an IC_{50} of 360 pM (Figure 11A). Taken together with the differential rank orders of binding affinities for omega-conopeptides at the two binding sites, as measured by displacement, these crosslinking experiments support the idea that the conopeptide binding components of site 1 and site 2 are distinct molecular entities, both with M_r 210-220 kDa.

3. Localization of Binding of Omega-conopeptides in Nervous Tissue

The omega-conopeptide binding sites described above are distributed differentially throughout the nervous system. The regional distribution of the binding sites and their relative affinities for the two conopeptides SNX-111 and SNX-183 in rat brain sections were determined by autoradiography of brain sections exposed to the radiolabelled compounds, detailed in Example 5. The results presented in Fig. 12 show that the distribution of binding of [^{125}I]-SNX-111 is highly localized (A, B) and that non-specific binding is virtually non-existent (C, D). The pattern of binding is similar to that reported using [^{125}I]-GVIA

parations (Takemura). Comparison of the specific ding of [^{125}I]-SNX-111 and [^{125}I]-SNX-183 revealed clapping but differential distribution of binding es (E, F). Both ligands labeled the cortex, CA1, tate gyrus and caudate-putamen. In these regions, ding of [^{125}I]-SNX-183 was unaffected by centrations of SNX-111 which caused complete placement of [^{125}I]-SNX-111 labeling (not shown), gesting colocalization of sites 1 and 2. Greater dance of site 2 in thalamic ventromedial lateral and medial geniculate was revealed by the high ity of binding of [^{125}I]-SNX-183 in these nuclei. ontrast, globus pallidus, CA_3 and substantia nigra labeled only by [^{125}I]-SNX-111, indicating a onderance of site 1 in these regions. The complete nce of [^{125}I]-SNX-183 binding in the substantia a suggests a third, distinct binding site gnized only by [^{125}I]-SNX-111, and by implication, el calcium channel subtype.

Computer-aided densitometric analysis of the acement of [^{125}I]-SNX-111 by SNX-183 in the erent brain regions showed that the labeling of ex and hippocampus by [^{125}I]-SNX-111 could be ited by lower concentrations of SNX-183 (IC_{50} 100 whereas higher concentrations of SNX-183 (IC_{50} 300 were needed to displace [^{125}I]-SNX-111 from the antia nigra. Since a number of nuclei that are to be rich in synapses and thus likely to contain n density of presynaptic calcium channels were not ed by either ligand, the two conopeptides can nguish four different subtypes of neuronal binding as summarized in Table 4. The four subtypes those sensitive to both SNX-111 and SNX-183 (site hose sensitive to SNX-183 only, (site 2), those nized by SNX-111 only and others recognized by

neither conopeptide.

Table 4

Four classes of OCT binding site calcium channels

Site	Binds SNX-111	Binds SNX-183	Examples
1	+	+	cortex, hippocampal CA1, CA3, thalamic nuclei, spinal cord (laminae I+II only)
2	-	+	cortex, hippocampal CA1, CA3, thalamic nuclei
3	-	-	midbrain nuclei, spinal grey matter (except laminae I + II), neuromuscular junction
4	+	-	substantia nigra, hippocampal CA2

4. Selective Inhibition of Neurotransmitter Release

Omega-conopeptides inhibit neurotransmitter release in various regions of the nervous system. As shown below, such inhibition varies according to the neurotransmitter, the omega-conopeptide, and the region studied. Neurotransmitters which can be measured, in accordance with various aspects of the invention, include, but are not limited to dopamine, norepinephrine, acetylcholine, GABA, glutamate, and a number of peptide neurotransmitters, such as substance P (McGeer).

Quantitation of release and inhibition thereof is determined by sensitive detection methods, also known in the art, including direct detection of release of

endogenous stores by HPLC or specific radioimmunoassay (RIA), and detection of release of pre-loaded, labeled compound. Alternatively, or in addition, detection of release may be achieved using a number of indirect assays, exemplified by the electrophysiological studies described above, in which whole tissue response to electrical or chemical stimulation is measured.

Inhibition of release of the neurotransmitter norepinephrine from neuronal cells can be assayed in mammalian brain hippocampal slices by standard methods, such as detailed in Example 6A. Briefly, hippocampal slices are distributed to individual wells of a microtiter plate, and incubated with radiolabeled norepinephrine under conditions favoring cellular uptake of the transmitter. The cells are washed with low-potassium medium, then bathed for 15 minutes in high-potassium stimulation medium, in the presence of selected concentrations of a test compound. After removal of the stimulation buffer, radioactivity remaining in each slice is determined.

Figure 13A shows concentration dependence of inhibition of norepinephrine release from hippocampal slices, as detailed in Example 8A. Basal (open bars) and potassium-stimulated (solid bars) release is shown in the presence of varying concentrations of SNX-111, as indicated.

Fig. 13B shows the effects of the three peptides SNX-111, SNX-183 and SNX-230 on the release of norepinephrine evoked by potassium depolarization. SNX-111 inhibits release with high potency (IC_{50} ≈ 1 nM) but only partially (approx. 60%). SNX-183 is much less potent (IC_{50} ≈ 180 nM) but the inhibition is substantially 100%. SNX-230 also inhibits release 100%, but in a biphasic manner, inhibiting

approximately 50% with high potency (IC_{50} = 0.02 nM) and 50% with much lower potency (IC_{50} = 65 nM). In agreement with the binding studies discussed above, these results imply that such norepinephrine release is mediated by at least two distinct subtypes of presynaptic calcium channels, one of which corresponds to the site 1 receptor identified by high affinity for SNX-111 and the other to the site 2 receptor recognized preferentially by SNX-230.

The IC_{50} values given in Table 5 for a variety of omega-conopeptides which have been examined by this method represent the average IC_{50} values calculated from thin (200 μ) and thick (400 μ) hippocampal slices. The three lowest IC_{50} values, between 0.8 and 2.4 nM, correspond to omega-conopeptides which are most potent in this assay.

Table 5

Inhibition of Norepinephrine Release by Omega-conopeptides

omega-conopeptides	IC_{50} (nM)
GVIA (SNX-124)	0.8
MVIIA (SNX-111)	1.5
TVIA (SNX-185)	2.4
SNX-201	11
SNX-195	11
SNX-202	29
SVIB (SNX-183)	200
SNX-191	>100
SVIA (SNX-157)	>4500

The selective effect of omega-conopeptides in inhibiting neurotransmitter release from specific types

of nerve terminals is demonstrated by the markedly different responses of other neurotransmitter systems, when exposed to omega-conopeptides. When tested in a synaptosomal preparation prepared from the neuronal rich electric organ of electric eel (Ommata dyscopyge), a different rank order of potency was observed (Table 6).

Table 6

Inhibition of Release of ATP from Electric Organ Synaptosomes

Compound	IC_{50}
SNX-111	42
SNX-195	84
SNX-183	1,700
SNX-185	>6,000
SNX-157	>6,000

Similarly, inhibition of release of amino acid neurotransmitters GABA and glutamate from rat neuronal tissue did not parallel either binding potency at the MVIIA site 1, the SVIB site 2 or inhibition of norepinephrine release (Table 7).

Table 7

Inhibition of release of GABA and Glutamate from rat Synaptosomes

Compound	IC_{50} (GABA)	IC_{50} (Glutamate)
SNX-185	100 nM	>100 nM
SNX-183	200 nM	200 nM
SNX-111	>200 nM	>200 nM

Effects of omega-conopeptides were also compared to those of OCT GVIA and amiodipine, an L-channel blocker, on potassium-stimulated release of dopamine

and acetylcholine from slices of rat brain (striatal region) as described in Example 6 (C,D). Briefly, in these experiments, striatal slices from rat brain were preloaded with radiolabelled dopamine or choline, then perfused for 45 minutes with bathing media. Slices were subjected to an S1 stimulus, consisting of addition of 15 mM potassium chloride to the bathing medium for 1 minute. Total outflow of radiolabeled neurotransmitter in response to S1 was measured. Slices were then washed, exposed to test compound for 20 minutes, then subjected to an S2 stimulus, as above. Comparison of outflow of neurotransmitter in response to S2 to outflow in response to S1 is a measure of drug effects on the system. Results are given as percent inhibition of release in Tables 8 and 9.

Table 8

Effect of Omega-conopeptides and amiodipine on [^{3}H] dopamine release from striatal slices

Compound	Concentration	% inhibition
GVIA	1 nM	5
	10 nM	52
MVIIA	1 nM	6
	10 nM	49
Amiodipine	1000 nM	0

Table 9

Effect of Omega-conopeptides and amiodipine on [^{3}H] acetylcholine release from striatal slices

Compound	Concentration	% inhibition
GVIA	3 nM	50
MVIIA	5.5 nM	50
Amiodipine	1000 nM	0

Further means of measuring inhibition of neuronal transmitter release are isolated tissue assays, such as atrial strip, aorta, vas deferens and guinea pig ileum assays, in which the response to a stimulus, usually an electrical stimulus, is correlated to the amount of neurotransmitter released from neurons innervating the tissue (Kenakin). In the guinea pig ileum, inhibition of electrically stimulated contractions is correlated with inhibition of acetylcholine release, as demonstrated by the ability of cholinergic agonists to overcome such inhibition. Example 7E describes the preparation and assay in detail. Table 10 shows the IC_{50} values for various omega-conopeptides on contraction of guinea pig ileum in response to electrical stimulation.

Table 10

Effects of conopeptides on electrically stimulated contraction of Guinea pig ileum

Compound	IC_{50} (nM)
SNX-111	13
SNX-185	29
SNX-183	91
SNX-157	>100

II. Treatment of Pain

In accordance with one aspect of the invention, it has been discovered that omega-conopeptides TVIA (SNX-185) or MVIIA (SNX-111), or derivatives thereof which are effective (a) to inhibit voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the peptide's ability to inhibit electrically stimulated contraction of the guinea pig ileum, and (b) to bind to omega conopeptide MVIIA binding sites present in neuronal tissue, are effective to produce

analgesia and to enhance the analgesic effect of an opiate compound.

A. Omega-conopeptides

Omega-conopeptides useful in the treatment of pain have been found, in accordance with the invention, to conform to certain physical and chemical constraints, as described below. Generally, omega-conopeptides useful in the treatment methods are those which are 25-35 amino acids in length and which have three disulfide bonds at specified positions along their length.

Based on a sequence homology analysis of the peptides whose full sequences are known (Figure 1), the naturally occurring active omega-conopeptides were grouped into distinct groups I and II, each with internal homologies distinct to that group, as can be appreciated from Figure 14. Group I includes active omega-conopeptides MVIIA (SNX-111) and MVIIB (SNX-159) which possesses a binding constant to the MVIIA site within the range of compounds showing activity in treating pain. Group II includes TVIA (SNX-185), SNX-207 and SNX-236. A third group includes inactive peptides SNX-231, and SVIA (SNX-157) and omega-conopeptides whose binding activities for the MVIIA site on neuronal membranes and/or activity in norepinephrine inhibition are outside the range of active compounds.

The three groups of omega-conopeptides are arranged in Figure 14 with their six Cys residues aligned, which places these residues at positions 1, 8, 15, 16, 20, and 28. To make this alignment, gaps were introduced at the positions shown in the three groups. In the analysis below, these gaps retain the assigned number shown in Figure 14, even though they represent amino acid deletions in the respective groups of active

omega-conopeptides.

Sequence variation in the peptides, based on primary structure alone, was analyzed by adopting the following constraints:

1. The peptides in both active groups (I and II) include the Cys residues at position 1, 8, 15, 16, 20, and 28. Other Cys residues could be substituted at the positions indicated below only if they are selectively protected during oxidation of the peptide to form the three disulfide linkages.

2. The peptides in the active groups include three disulfide linkages connecting the Cys residues at positions 1 and 16, 8 and 20, and 15 and 28. As described above, the disulfide bridges are formed by air oxidation of the full sequence peptide in the presence of DTT. The ability of the peptide to form the three desired disulfide linkages would therefore require that the peptide, prior to disulfide bridging, be able to adopt a conformation which allows the three selected linkages, with or without the Cys protecting-group strategy discussed above. This constraint would thus exclude amino acid variations which prevent or otherwise hinder the formation of the three selected bridges.

Constraints 1 and 2 preserve the basic conformation of the omega-conopeptides imposed by the three disulfide bridges.

3. Within Group I, the amino acid variations which occur at the six non-conserved residues are allowed, including peptides in which the carboxy terminus is amidated or has a free acid form. That is, the first group compound derivatives include the peptide structures having the form: SEQ ID NO: 22-X_1-SEQ ID NO: 23-X_2-SEQ ID NO: 25-X_3X_4-SEQ ID NO: 24-X_5-SEQ ID NO: 25-X_6-SEQ ID NO: 26-t, where X_1=K or S; X_2=S or

H; X_3=L or T; X_4=M or S; X_5= N or a deletion; X_6=S or deletion, and t= a carboxy or amidated carboxyterminal group, and where SEQ ID NO: 22 is C K G K G A; SEQ ID NO: 23 is C; SEQ ID NO: 25 is R; SEQ ID NO: 24 is Y D C C T G S C; and SEQ ID NO: 26 is G K C.

4. Within Group II, the amino acid variations which occur at the eight non-conserved residues are allowed, including peptides in which the carboxy terminus is amidated or has a free acid form. Thus, the second group compound derivatives include the peptide structures having the form: SEQ ID NO: 27-$X_1X_2X_3$-SEQ ID NO: 28-X_4-SEQ ID NO: 29-t, where X_1=X or R; X_2=T or L; X_3= S or M, X_4= X or P; and t= a carboxy or amidated carboxyterminal group, and where SEQ ID NO: 27 is C L S X G S S C S; SEQ ID NO: 28 is Y N C C R S C N; and SEQ ID NO: 29 is Y S R K C R.

5. Considering both active groups together, amino acid positions which are conserved in all active species are preserved. Thus, for example, the Cys residues, the 5-position glycine, the 13-position tyrosine, the 19-position serine, and the 26-position lysine are all preserved. Preferred OCT analogs or derivatives may be selected by comparing, for purposes of inter-sequence conservation and substitution, those sequences known to be active. For example, in the case of the treatment of pain, omega-conopeptides MVIIA (SNX-111) and TVIA (SNX-185) are known active compounds. Active derivatives are those peptides having, in addition to the conserved cysteine residues described above, a conserved glycine residue at position 5, conserved serine residues at positions 9, 19, and 24, and a conserved lysine residue at position 26. Inter-sequence substitution of variable residues is then preferable in the formation of active analogs. For example, analog position 2 may be occupied by a

lysine or a leucine residue, and position 6 may be occupied by an alanine or a serine residue.

6. Considering both active groups together, there are amino acid positions which are likely to be variable within the range of active species. For example, the position 2 amino acid may be lysine or leucine, the position-3 amino acid may be glycine or serine, and the position 4 amino acid, hydroxyproline or arginine. In addition, if the two or more amino acids at a variant position are in a common substitution class, substitution within that class may be favorable. Standard substitution classes are the six classes based on common side chain properties and highest frequency of substitution in homologous proteins in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix (Dayhoff). These classes are Class I: Cys; Class II: Ser, Thr, Pro, Hyp, Ala, and Gly, representing small aliphatic side chains and OH-group side chains; Class III: Asn, Asp, Glu, and Gln, representing neutral and negatively charged side chains capable of forming hydrogen bonds; Class IV: His, Arg, and Lys, representing basic polar side chains; Class V: Ile, Val, and Leu, representing branched aliphatic side chains, and Met; and Class VI: Phe, Tyr, and Trp, representing aromatic side chains. In addition, each group may include related amino acid analogs, such as ornithine, homoarginine, N-methyl lysine, dimethyl lysine, or trimethyl-lysine in class IV, and a halogenated tyrosine in Group VI. Further, the classes may include both L and D stereoisomers, although L-amino acids are preferred for substitutions.

7. Considering the known inactive species, substitutions to amino acids which are present in inactive species, but not active ones, at any selected residue position, are not favored to preserve activity

in the active compounds. Thus, for example, although a 3-position serine is present in both active and inactive compounds, 4-position serine or threonine is present in inactive species only, and either substitution is thus disfavored.

The above amino acid selection rules 6-7 are intended as a guide for allowed amino acid substitutions within active omega-conopeptides. Once an amino acid substitution or modification is made, the peptide is further screened for the requisite calcium channel antagonist activity, and the requisite activities for inhibition of neurotransmitter release and binding to the appropriate OCT binding site of neuronal membranes, as described above.

Several of the amino acid substitutions or modifications to the omega-conopeptide illustrate the principles outlined above.

Omega-conopeptides which are selected on the basis of these criteria, discussed in detail below, are tested for ability to produce or enhance analgesic effects produced by sub-maximal doses of opioid compounds in a standard test of analgesia, such as the Rat Tail-Flick test, wherein analgesia is measured by a prolongation of reaction time to a noxious radiant heat stimulus.

B. <u>In vitro Properties of Analgesic Omega-conopeptides</u>

1. <u>Calcium channel blocking activity</u>. Calcium channel blocking activity was measured electrophysiologically in neuronal (N1E-115 or IMR-32) cell lines, as described in Section II, above, and in detail in Example 1. Omega-conopeptides having calcium channel blocking activity are those which block calcium currents in such cell lines with potencies in the range observed for omega-conopeptides MVIIA and GVIA in N1E-

115 cells, or displaying the efficacy observed for omega-conopeptides MVIIA and SVIB in IMR-32 cells (Figure 5C).

2. High Affinity Binding to OCT Binding Sites. Methods for determination of binding affinity to OCT binding sites are discussed in Examples 2-4, below.

Experiments testing reversibility of binding of SNX-111, SNX-183 and SNX-124 revealed that SNX-111 and SNX-183 exhibited dissociation half-times of two and five minutes, respectively. In contrast, SNX-124 did not dissociate appreciably from its binding site(s), even 1 hour following addition of excess unlabelled compound.

Compounds were tested for their ability to displace binding of SNX-111 or SNX-183 from their respective binding sites. In displacing SNX-111, it was found that compounds which produce or enhance opioid anti-nociceptive activity, such as OCT MVIIA (SNX-111), and TVIA (SNX-185), have IC_{50} values between about 15 and 300 pM, and K_i values between about 1 and 100pM. In contrast inactive compound SNX-183 had an IC_{50} of greater than 1000 pM for binding at the MVIIA site.

From the foregoing, it is seen that active compounds in accordance with the invention are characterized by a high binding affinity for MVIIA binding site 1. The binding affinity for these sites may be characterized as follows. In the first approach, the binding affinity of the compound for the MVIIA site, as estimated by IC_{50} at the site, is compared directly with those of selected high affinity active compounds, such as SNX-111 and SNX-185. An active compound is one whose binding affinity is at least as high as and preferably within the range of binding affinities measured for such high affinity

OCT's. Secondly, the binding affinity of the test compound can be characterized by binding to SVIB binding site 2, as described above for binding to MVIIA binding site 1. Thirdly, the binding affinity of the compound can be characterized by the ratio of binding constants or relative potencies of the compound for the MVIIA and SVIB sites, as just described. Here an active compound is one whose binding ratio is within the range for the selected active peptides, such as MVIIA (SNX-111) and TVIA (SNX-185); i.e., the binding ratio is substantially within the range of the ratio observed for the omega-conopeptides MVIIA and TVIA.

A number of omega-conopeptide compounds which were tested gave IC_{50} and K_i values lower than or within the ranges of those of omega-conopeptides MVIIA (SNX-111) and TVIA (SNX-185) for binding at the SNX-111 site, and these compounds should thus be considered candidates as anti-nociceptive enhancing compounds. However, some of these compounds, may not fulfill additional criteria for anti-nociceptive compounds of the invention, as described herein.

3. Localization of OCT binding in the central nervous system. Conopeptide SNX-111 binds to distinct regions of the brain and spinal cord which are commonly associated with pain pathways (Figure 12). These include the periaquaductal grey (PAG) region of the brain and the dorsal horn of the spinal cord. The distribution of CgTx (GVIA) binding shown by Takemura et al (1989) also shows localization of CgTx binding sites at a very high level in the dorsal horn of the spinal cord (1st and 2nd layers of Rexed) and, to a lesser degree, in the central grey region of the mesencephalon, which may correspond to the PAG; however, some of these compounds may not fulfill

additional criteria for antinociceptive compounds of the invention, as described herein.

4. Inhibition of neurotransmitter release. Another requisite property of analgesic, anti-nociceptive OCT compounds, in accordance with the invention, is their ability to specifically inhibit depolarization-evoked and calcium-dependent neurotransmitter release from neurons. In the case of anti-nociceptive omega-conopeptides, inhibition of electrically stimulated release of acetylcholine at the myenteric plexus of the guinea pig ileum (Example 6E) is predictive of anti-nociceptive activity, as seen in Table 10. Omega-conopeptides having anti-nociceptive activity and/or anti-nociceptive enhancing activity have IC_{50}'s in the range of those values observed for active omega-conopeptides MVIIA (SNX-111) and TVIA (SNX-185), or less than approximately 50 nM, as shown in this assay.

5. In vivo measurements of analgesia. Analgesia is conveniently measured in one or more of a number of animal models, in which an animal's response to a given pain stimulus is measured. One such model is the Rat Tail-Flick test, described in Example 7. Briefly, in this test, a rat is positioned such that its tail is exposed to a standard heat source, and the time that the animal voluntarily endures the heat, prior to moving its tail, is recorded. Analgesics, particularly opioid analgesics, prolong this time.

Shown in Figure 15 are the results of experiments in which the effects of a sub-maximal dose of morphine were compared to those of the combination of a sub-maximal dose of morphine and a 0.5 μg (intrathecal) dose of SNX-185 in the Rat Tail-Flick Test. Animals were tested at various time points following injection,

as indicated, and latency of tail-flick recorded. In addition, for each animal, the maximal latency response was recorded, and the mean of such maximal responses calculated as the maximal percent effect (MPE). Intrathecal administration of SNX-185 enhanced the effects of a sub-maximal dose of morphine (Figure 15) in this assay at all time points, and significantly at 45 min. after administration of compound. By the term "submaximal dose" is meant a dose of morphine or other opiate which is insufficient to induce maximal latency of response in the tail-flick assay, measured as Percent Effect, the calculation of which is described in Example 7.

Table 11 shows the effects of SNX-185 given alone or in combination with varying doses of morphine, shown on the right side of the table. SNX-185 showed a significant analgesic effect when given alone at a dose of 0.5 μg, in at least one of the trials. When given in conjunction with a low dose (0.05 μg) of morphine, 0.5 μg SNX-185 yielded a significant enhancement of the effect of morphine alone.

In separate experiments (Table 12), SNX-111 (0.1 μg) enhanced the effects of sub-maximal doses of morphine, whereas SNX-124 (0.1 μg) had no effect. SNX-183 (1 μg) showed a small but measurable effect, but this effect was not significant when compared to control. SNX-236, a TVIA-derivative shown in Figure 2 was also found to be analgesic in the Rat tail-flick assay.

Table 11

Effect of SNX-185 ± Morphine on Latency Percent Effect in Rat Tail-Flick Assay

SNX-185 Dose (μg)	n	Time After Injection (min) % Effect (± SE) 25	45	Morphine Dose (μg)	Time After Injection (min) % Effect (± SE) 25	45
0	6	20 ± 16	18 ± 12	0.2	58 ± 22	66 ± 17
0.5	7	29 ± 13	*57 ± 13	0.2	98 ± 2	92 ± 5
0	8	16 ± 15	12 ± 14	0.1	61 ± 15	75 ± 11
0.5	9	0 ± 4	13 ± 11	0.1	60 ± 17	51 ± 17
0	5	9 ± 5	8 ± 3	0.1	45 ± 20	49 ± 16
0.5	5	12 ± 10	8 ± 3	0.1	79 ± 21	77 ± 21
1.0	4	33 ± 22	36 ± 20	0.1	-	-
0	8	1 ± 3	5 ± 2	0.05	7 ± 6	16 ± 11
0.5	8	*27 ± 11	14 ± 7	0.05	*44 ± 16	30 ± 18

*p < .05 vs. Control.

Table 12

Effect of Coadministration of Conopeptides and Morphine on Analgesia (% Effect) in Rat Tail-Flick Assay

	Time After Injection 15	30	45	MPE
Morphine, 0.05 μg (n=14)				
Mean	15	13	7	23
SE	7	6	5	8
SNX-111, 0.1 μg + morphine (n=13)				
Mean	12	33	*38	42
SE	9	10	12	11
SNX-124, 0.1 μg + morphine (n=14)				
Mean	*-7	5	11	14
SE	5	6	11	8
SNX-183, 1.0 μg + morphine (n=14)				
Mean	18	31	25	39
SE	6	8	6	7

* p < .05 vs. Morphine

An in vivo test of analgesic potency is the rat formalin test. Briefly, in this test, a standard dose of formalin is injected into the rat paw, and flexions of the paw are quantitated. Typically in this assay a biphasic response pattern is observed, with numerous responses observed during the period 5 min. after injection (Phase 1) and a second phase (Phase 2) which occurs during the period about 10-60 minutes following injection (Fig. 16). Quantitation of responses during each phase is made by calculation of area under the curve of flinches/min. as described in Example 8.

Figure 17 shows results of experiments in which SNX-111, SNX-185 and SNX-231 were tested for effects on the formalin response in rats. From the curves shown, doses which resulted in approximately 50% inhibition in Phase 1 and Phase 2 responses were determined (Table 13). As shown in Figure 16, administration of SNX-111 and SNX-185 each resulted in dose dependent inhibition of both Phase 1 and Phase 2 response evoked by formalin. SNX-231 was without effect at the doses employed in the assay.

Table 13

ED_{50} Values for Intrathecal Administration of Conopeptides on Phase 1 and Phase 2 of the Formalin Test

Drug	N	Phase 1	Phase 2
SNX-111	21	0.009 μg	0.013 μg
SNX-185	20	0.02 μg	0.05 μg
SNX-231	12	>1.0 μg	>1.0 μg

Analgesic potency of conopeptides can also be tested in animal models of neuropathic or neurogenic

pain. One such model resembles the human condition termed causalgia or reflex sympathetic dystrophy (RSD) secondary to injury of a peripheral nerve. This condition is characterized by hyperesthesia (enhanced sensitivity to a natural stimulus), hyperalgesia (abnormal sensitivity to pain), allodynia (widespread tenderness, characterized by hypersensitivity to tactile stimuli), and spontaneous burning pain. In humans, the pain induced by neuropathy tends to be chronic and may be debilitating. Treatment may involve repeated sympathetic ganglionic blockade. Such blockade can be carried out surgically or using direct application of drugs, such as anesthetics or morphine, even when such drugs are ineffective systemically. In accordance with the invention, analgesic omega conotoxin peptides are effective in providing relief of neuropathic pain, as described below.

Experiments carried out in support of the present invention were performed in a rat model of peripheral neuropathy detailed in Example 9. Briefly, in the model used, rats are subjected to a surgical procedure, described by Kim et al. and Bennett et al., designed to reproducibly injure peripheral nerves (spinal nerves L5 and L6). These rats develop a hyperesthetic state, which can be measured, using one or more paradigms known in the art. Here, allodynia was measured by stimulation of neuropathic rat hindlimb using wire hairs having graded degrees of stiffness. Analgesic compounds reverse the heightened sensitivity such animals exhibit to the stimulus.

Results of animals treated with saline, 0.3, 1, or 3 μg of SNX-111 are shown in Figures 18 and 19. Data are expressed as percent maximum effect, where the maximum effect indicates a complete reversal of surgically induced allodynia, or relative insensitivity

to stimulus (maximum equals 15 gram hair stimulus). A baseline of zero indicates a mean sensitivity to a wire hair graded at less than 3 grams. As shown in Figure 18, treatment of rats (n=6/treatment) with 1 or 3 μg SNX-111 resulted in elevation of threshold response. Peak elevation of response due to drug treatment (reversal of allodynia) was observed by 30-60 minutes, and effects lasted in excess of 60 minutes.

Figure 19 summarizes and shows statistical analyses, as detailed in Example 9, of the data shown in Figure 18. Also shown in Figure 18 is the response of animals treated with 10 μg morphine sulfate. In the study shown, treatment of rats with 3 μg SNX-111 or 10 μg morphine resulted in significant reversal of allodynia in comparison to saline treated animals.

These results indicate that analgesic omega conotoxin peptides, exemplified by SNX-111, are capable of reversing the hyperesthetic effects induced by nerve damage.

III. Summary

As indicated above, conopeptides such as MVIIA and TVIA, and their derivatives, have a number of peptide-specific binding/inhibitory activities, which include:

(1) high-affinity binding to the MVIIA binding site of neuronal cells;

(2) inhibition of norepinephrine release selectively in central nervous system neuronal cells;

(3) inhibition of voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the inhibition of electrically stimulated contraction of the guinea pig ileum; and

(4) Inhibition (blockage) of membrane currents associated with N-type or omega HVA neuronal calcium channels in an isolated cell system, such as the mouse

neuroblastoma cell line;

Previously it has been shown (co-owned U.S. Patent No. 5,051,403) that conopeptides having defined binding/ inhibitory activities are effective in reducing neuronal damage related to an ischemic condition in mammals. The binding/inhibitory activities of conopeptides effective in such treatment include:

(a) high-affinity binding to the MVIIA binding site; and

(b) selective inhibition of norepinephrine release in central nervous system neuronal cells.

Two conopeptides which have these characteristic activities, and which have been shown effective in reducing post-ischemia neuronal damage, are conopeptides MVIIA and TVIA.

In the Section above, it was shown that conopeptides, such as MVIIA and TVIA, which have defined binding/inhibitory activities, are effective in producing analgesia and in enhancing analgesia by opiates. The important binding/inhibitory activities are:

(a) high-affinity binding to the MVIIA binding site; and

(b) inhibition of voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the inhibition of electrically stimulated contraction of the guinea pig ileum.

The following examples are intended to illustrate various characteristics of the method of the invention, but are in no way intended to limit the scope of the invention.

Example 1

Calcium-Channel Antagonist Activity: Inhibition of Ionic Currents

Ionic currents through calcium channels were examined in cells that were voltage-clamped by a single patch-clamp electrode. These whole-cell patch-clamp studies were performed mainly on N1E115 mouse neuroblastoma cells, although a variety of cell types, including human neuroblastoma cell line IMR-32, have been examined.

A. Current Measurement Methods

Most measurements were obtained using a bath saline that allowed examination of the calcium currents in the absence of other ionic currents. These solutions contained 80 mM NMDG (as a sodium replacement), 30 mM TEACl (to block potassium currents), 10 mM $BaCl_2$ (as a charge-carrier through the calcium channels), and 10 mM HEPES at pH 7.3. Some solutions also contained 2 mM quinidine (to block potassium currents) and 3 μM tetrodotoxin (to block sodium currents). Normal bath saline was (mM): 140 NaCl, 10 glucose, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10mM HEPES pH 7.3. Intracellular solutions contained 150 mM CsCl, 0.5 mM $CaCl_2$, 5 mM EGTA, 5 mM $MgCl_2$, 2 mM K_2ATP at pH 7.3-7.4. Bath saline and all internal solutions were filtered before use.

Pipets were made from Corning 7052 glass (Garner Glass Company, Claremont, CA 91711), coated with Sylgard (Dow Corning, Midland, MI 48640) and fire-polished before use. Bubble numbers were typically 5 to 6, with pipet resistances typically 2-5 MOhms. Corning 8161, Kimble, and other glasses were also used without noticeable effect on the calcium currents observed.

Recordings were carried out at room temperature with an Axopatch 1-C amplifier (Axon Instruments, Foster City, CA 94404) and analyzed with pCLAMP software (Axon Instruments). Data were filtered at 1000 Hz for a typical sampling rate of .1 kHz; in all cases data were filtered at a frequency at most 1/5 of the sampling rate to avoid biasing. Data were collected on-line by the software. Analysis was performed on-screen with print-out via a Hewlett-Packard LaserJet Printer (Hewlett-Packard, Palo Alto, CA 94306).

The typical experiment was conducted as follows: after seal formation followed by series resistance compensation and capacitative transient cancellation, a voltage clamp protocol was performed wherein the cell potential was stepped from the holding potential (typically -100 mV) to test potentials that ranged from -60 mV to +20 mV in 10 mV increments. The cell was held at the holding potential for 5 seconds between pulses. Protocols starting from other holding potentials usually covered the same range of test potentials.

B. Current Inhibition Measurement

Figure 3 shows calcium current traces from an N1E-115 mouse neuroblastoma cell. The figure is read from left to right in time, with downward deflections of the trace indicating positive current flowing into the cell. Currents were elicited by a voltage step from 100 mV to -10 mV. The cell was bathed in saline with sodium replaced by NMDG and 10 mM Ba^{++} instead of 2 mM Ca^{++}. Potassium currents were blocked by TEA in the bath and Cs^{+} in the pipet solution.

The three traces in Figure 3, labeled B-D, show decreasing calcium currents, with increasing MVIIA omega-conopeptide concentrations of 10 nM (3B), 50 nM

(3C), and 200 nM (3D).

The response of voltage-gated calcium current to increasing dosages of OCTs MVIIA and GVIA are shown in Figure 4. The calculated IC_{50} is approximately 10 nM for GVIA and 100 nM for MVIIA. These values indicate extremely high specificity of the peptides for their site of action.

Table 1 compares IC_{50} values for GVIA, MVIIA, SVIB and SVIA OCTs. Whereas OCT GVIA and OCT MVIIA show 50% inhibition of the measured calcium current at nanomolar concentration range, IC_{50} values for OCT SVIB and OCT SVIA were not measurable within the range of concentrations tested, and are therefore listed as having IC_{50} values above the micromolar concentrations indicated.

Example 2

Synaptosomal Membrane Preparations

A. Mammalian-Brain Synaptosomes and Synaptosomal Membranes.

Synaptosomes were prepared from rat whole brain or hippocampal region of brain. Rats were sacrificed, and forebrains were removed and transferred to 10 ml ice-cold 0.32 M sucrose containing the following protease inhibitors (PI): 1 mM EGTA; 1 mM EDTA; 1 uM pepstatin; 2 uM leupeptin. Brains were homogenized using a motor-driven Teflon-glass homogenizer (approx. 8 passes at 400 rpm). Homogenates from 4 brains were pooled and centrifuged at 900 xg for 10 minutes at 4 degrees. Supernatants were then centrifuged at 8,500 xg for 15 minutes. Resulting pellets were resuspended in 10 ml each ice-cold 0.32 M sucrose plus PI with vortex mixing. The suspension was then centrifuged at 8,500 xg for 15 minutes. Pellets were resuspended in 20 ml ice-cold 0.32 M sucrose plus PI. The suspension (5

ml/tube) was layered over a 4-step sucrose density gradient (7ml each: 1.2 M sucrose, 1.0 M sucrose, 0.8 M sucrose, 0.6 M sucrose; all sucrose solutions containing PI). Gradient tubes were centrifuged in a swinging bucket rotor at 160,000 xg for 60 minutes at 4 degrees. The 1.0 M sucrose layer plus the interface between the 1.0 and 1.2 M sucrose layers were collected and diluted with ice cold deionized water plus PI to yield a final sucrose concentration of 0.32 M. The resulting suspension was centrifuged at 20,000 xg for 15 minutes. Pellets were then resuspended in 5 ml ice-cold phosphate buffered saline plus PI. The resulting rat brain synaptosomes were then aliquoted and stored in a liquid nitrogen containment system.

Prior to use in binding assays, synaptosomes were thawed and diluted with 3 volumes of ice cold deionized water plus PI. This suspension was homogenized using a PT 10-35 Polytron (setting 6) for two 10-second bursts. The homogenate was centrifuged at 40,000 xg for 20 minutes at 4 degrees. The resulting pellets were resuspended in about 5 ml of ice cold phosphate buffered saline plus PI. The resulting brain synaptosomal membrane preparation was aliquoted and stored at -80°C until use. Protein concentration of the membrane preparation was determined using Bradford reagent (BioRad), with bovine serum albumin as standard.

B. Electric organ synaptosomes

Electric organ synaptosomes were prepared by dissection from marine electric rays (Ommata dyscopyge or Narcine brasiliensis) that had been stunned with 0.25 g/liter tricaine HCl and cooled to 4°C immediately prior to dissection. All subsequent manipulations were carried out at 0-4°C whenever possible. Organs were diced and homogenized for 4 15-second periods in a

Waring blender with an equal weight of synaptosome buffer (SB) (20 mM HEPES, Ph 7.2, 280 mM NaCl, 3mM KCl, 1.8 mM $MgCl_2$, 300 mM urea, 100 mM sucrose, 5.5 mM glucose plus protease inhibitors), (1 mM EGTA, 1 μM pepstatin, 2 μM leupeptin, 1 μg/ml aprotinin and 0.1 mg/ml bacitracin).

The homogenate was filtered through cheesecloth and centrifuged at 30,000 x g for 15 min. The supernatant was discarded and each pellet was taken up in 10 ml synaptosome buffer plus protease inhibitors. The resuspended pellets were combined and further disrupted with 5 strokes of a Teflon pestle in a glass homogenizer set at 400 rpm. The resulting suspension was centrifuged at 30,000 x g for 15 min. The supernatant was discarded and the pellet resuspended in approximately 5 ml of SB with protease inhibitors using a Teflon-glass homogenizer. This homogenate was layered onto six 32 ml 3-20% Ficoll gradients in SB (no protease inhibitors) and centrifuged at 100,000 x g for 1 hour in a swinging bucket rotor. The synaptosome band (the first band below the buffer-gradient interface) of each gradient was aspirated off and diluted 2:1 with synaptosome buffer with protease inhibitors. The diluted synaptosome suspension was pelleted at 30,000 x g for 15 min and resuspended in synaptosome buffer and refrigerated, for use in ATP release assays within 2-3 days of preparation. For binding experiments, aliquots were frozen at -160°.

Example 3

Omega-conopeptide Binding to Omega-conopeptide Binding Sites in Synaptosomal Membranes

A. Saturation Binding Assay

MVIIA OCT was radiolabeled with ^{125}I-iodine by

reaction with Iodogen™, essentially according to the method of Ahmad and Miljanich. Following the Iodogen reaction, the peptide solution was chromatographed by HPLC through a C-8 reversed phase column and eluted with a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in water/acetonitrile (40:60 vol/vol). The major peak of radioactivity following the underivatized MVIIA OCT was collected.

The binding constant (K_d) for [^{125}I]-MVIIA OCT to rat brain synaptosomal membranes was determined by a saturation binding method in which increasing quantities of [^{125}I] MVIIA OCT were added to aliquots of a synaptosomal membrane preparation (10 ug membrane protein, suspended in binding buffer consisting of 20 mM HEPES, pH 7.0, 75 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 2μM leupeptin, .035 μg/ml aprotinin, and 0.1% bovine serum albumin (BSA), in a total volume of 0.5 ml). Binding at each concentration of labeled compound was determined in the absence and presence of 1 nM unlabeled MVIIA OCT to determine specific binding (as described in part B, below). The amount of labeled peptide specifically bound at each concentration was used to determine B_{max}, the concentration of specific binding sites on the synaptosomes, and K_d, following standard binding analysis methods (Bennett). Figure 6A shows a saturation binding curve of [^{125}I]MVIIA to rat synaptosomal membranes. Figure 6B shows a Scatchard transformation of the data, from which a calculated K_d of about 10 pM is determined.

B. Reversibility of Binding

Rat brain synaptosomal membranes were incubated with a concentration of radiolabeled ligand approximating the K_d of the ligand for its binding site, for a period of time sufficient to achieve equilibrium binding. A high concentration of unlabeled

ligand was then added to the mixture, and the incubation continued. At time intervals, samples of the mixture were tested for binding of radiolabeled compound. As shown in Figure 7, SNX-111 exhibited reversible binding with a dissociation half-time of about 2 min. Likewise, SNX-183 binding exhibited reversible binding with a dissociation half-time of about 5 min. In contrast, radiolabeled SNX-124 showed no dissociation from its binding site over the time period studied (60 min).

C. Competitive Displacement Binding Assay

1. Competitive Displacement of OCT MVIIA

Rat brain synaptosomal membranes prepared as described in Example 2 were suspended in a binding buffer consisting of 20 mM HEPES, pH 7.0, 75 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 2μM leupeptin, .035 μg/ml aprotinin, and 0.1% bovine serum albumin (BSA). [^{125}I]-MVIIA (SNX-111) OCT (25-30,000 cpm, approximately 1500-2000 Ci/mmol) and test compound were aliquoted into polypropylene tubes, in the absence or presence of 1 nM MVIIA (SNX-111) OCT to determine non-specific binding. The membrane suspension was diluted and aliquoted last into the test tubes, such that each assay tube contained 10 μg membrane protein and the total volume was 0.5 ml. After incubation for 1 hour at room temperature, tubes were placed in an ice bath, then filtered through GF/C filters (Whatman), which were pre-soaked in 0.6% polyethyleneimine and prewashed with wash buffer (20 mM HEPES, pH 7.0, 125 mM NaCl, 0.1% BSA) using a Millipore filtration system. Just prior to filtration, each assay tube received 3 ml ice-cold wash buffer. The filtered membranes were washed with two 3 ml volumes of ice-cold wash buffer, dried, and filter-bound radioactivity was measured in a Beckman

gamma counter (75% counting efficiency).

Representative displacement binding curves for rat brain synaptosomal membranes are illustrated in Figure 8. IC_{50} values were computed from line fit curves generated by a 4-parameter logistic function. These values represent the concentration of test compound required to inhibit by 50% the total specific binding of [^{125}I]-MVIIA (SNX-111) OCT to rat brain synaptosomal membranes, where specific binding is defined as the difference between binding of [^{125}I]-MVIIA (SNX-111) OCT in the absence and presence of excess (1 nM) unlabelled MVIIA OCT. Non-specific binding is that binding of radiolabeled compound which is measured in the presence of excess unlabeled MVIIA OCT. Such values serve as approximations of the relative affinities of a series of compounds for a specific binding site.

2. Competitive Displacement of OCT SVIB

Rat brain synaptosomal membranes were prepared as described in Example 3. OCT SVIB was radiolabeled by iodination with ^{125}I-iodine by the Iodogen reaction, described in Example 4. Displacement binding of radiolabeled SVIB on rat brain synaptosomal membranes was carried out as in Example 4B. SVIB displacement curves for several of the omega-conopeptides assayed is shown in Figure 9. IC_{50} values and relative potency values were calculated as described below. Table 4 shows the relative potency values for omega-conopeptides examined, and the ratio of relative potencies of the compounds for the OCT MVIIA site and to the SVIB binding site.

The binding constant (K_i) for each test substance was calculated using non-linear, least-squares regression analysis (Bennett & Yamamura) of competitive binding data from 2 assays performed in duplicate on separate occasions. The relationship between K_i and

IC_{50} (concentration at which 50% of labeled compound is displaced by test compound is expressed by the Cheng-Prusoff equation:

$$K_i = IC_{50}/(1 + [L]/K_d)$$

where IC_{50} is the concentration of test substance required to reduce specific binding of labeled ligand by 50%; [L] is the concentration of [^{125}I]-MVIIA (SNX-111) OCT used in the experiment; and K_d is the binding constant determined for binding of [^{125}I]-MVIIA (SNX-111) OCT to rat brain synaptosomal membranes in saturation binding experiments. Table 3 summarizes computed IC_{50} for various omega-conopeptides for the MVIIA binding site of rat brain synaptosomal membranes.

Relative potency for displacement of binding is calculated as a ratio of the IC_{50} of the test compound and the IC_{50} of the reference compound. The reference compound is generally the unlabeled equivalent of the labeled ligand. Calculation of relative potency is as follows:

$$[\log (\text{relative potency})] = \log (IC_{50(ref)}) - \log (IC_{50(test)})$$

Relative potency values for binding at OCT MVIIA (SNX-111) and OCT SVIB (SNX-183) sites are listed in Table 3.

Example 4

Crosslinking of [^{125}I]-SNX-111 and [^{125}I]-SNX-183 to their polypeptide receptors.

A. SDS-gel electrophoretic analysis of rat hippocampal synaptosomal membrane polypeptides chemically crosslinked with A. [^{125}I]-SNX-111 and B. [^{125}I]-SNX-183. Both radioactive ligands (1nM) were incubated with rat hippocampal synaptosomal membranes in the absence (middle lanes) or presence (right

lanes) of excess non-radioactive peptide at 1000 times the IC50 for binding (i.e., 10 nM for SNX-111 and 1 mM for SNX-183) and crosslinking was achieved by the addition of N-hydroxysuccinimide (NHS) and the water-soluble carbodiimide, EDC (45). The left lanes are controls to which EDC and NHS were not added.

B. Displacement of crosslinked ^{125}I-SNX-183 by SNX-111

[^{125}I]-SNX-111 (A) or [^{125}I]-SNX-183 (B) crosslinked to the 210kDa polypeptide(s) in rat hippocampal synaptosomal membranes were displaced by increasing concentrations of non-radioactive SNX-111 and SNX-183 (45). As expected, the site 1-specific ligand [^{125}I]-SNX-111 was displaced monotonically by both SNX-111 and SNX-183. In contrast, displacement of [^{125}I]-SNX-183 by SNX-111 is biphasic with IC50's similar to its IC50's for binding to site 1 and site 2. The amount of radioiodinated peptide incorporated into the 210kDa band of crosslinked hippocampal synaptosomal membranes exposed to varying concentrations of competing peptides was estimated by scanning densitometry. The optical density of the 210kDa band in the sample without added competing peptide was taken as 100%. Curves were fit to the data as described above.

Example 5

Localization of OCT binding sites in Neuronal Tissue by Receptor Autoradiography

Adult male (Fischer or Sprague-Dawley, 250-300 g) were euthanized with carbon-dioxide, and whole brains were dissected out of the skull and rapidly frozen in iso-pentane pre-cooled on frozen carbon dioxide. The frozen brains were stored at -80°C and used within a week.

Coronal sections (20 μ thick) were obtained by

slicing (at -10°C-15°C) through the frozen brain using a cryostat microtome. The sections were thaw-transferred onto glass slides precoated with gelatin. Glass slides with the frozen sections were stored at -80°C and used within a week. Binding of [^{125}I] MVIIA was performed at room temperature. Each brain section was incubated for 40 min. with 250 μl of binding buffer: (HEPES/NaOH (20 mM, pH 7.5), EDTA (0.1 mM), EDTA (0.1mm) leupeptin (2 μM), Aprotinin (0.63 mg/ml), 1.5% BSA (RIA Grade), and [^{125}I] MVIIA (100-150 pM). To determine the proportion of non-specific binding selected adjacent brain sections were incubated with an excess of unlabelled peptide (25 nm).

After the incubation, binding buffer was carefully poured onto blotting paper and the slides transferred to a glass slide holder. Unbound [^{125}I] MVIIA was washed away by serially passing the slides through four dishes of washing buffer at room temperature for a total washing time of 16 min. Washing buffer contained HEPES/NaCH (50 m19, pH 7.5), NaCl (170 mM), BSA (RIA grade 1g/L) and Triton X-100 (0.05%). After the final wash, the slides were dipped quickly five times in water and dried with a blow-dryer.

Dried slides were exposed to XAR-2 film, overnight at room temperature and developed. The developed images were examined wither directly or by computer assisted image analyzer. The assignment of binding to specific neuroanatomical sites was made using an anatomical atlas of rat brain (Paxinos).

Autoradiograms show the distributions of [^{125}I]-SNX-111 (A,B,C,D) and [^{125}I]-SNX-183 (E,F,G,H) binding to coronal rat brain sections. Labeling in the presence of excess non-radioactive SNX-111 (C,D)

or SNX-183 (G,H) shows that non-specific labeling is negligible. Rostral sections (A,C,E,G) and caudal sections (B,D,F,H) are each adjacent or near-adjacent. "CA" indicates the CA_3 region of the hippocampus and "SN" indicates the substantia nigra.

Example 6

Inhibition of Neurotransmitter Release

A. Inhibition of Norepinephrine release

Inhibitory constants (IC50's) reflecting the potency of SNX-111 and SNX-183, for blocking the K^+-evoked release of exogenous, loaded [^{3}H]-norepinephrine from rat hippocampal slices were determined. Freshly dissected hippocampal slices in oxygenated buffered saline were loaded with [3H]-norepinephrine and washed three times. Slices were then exposed to buffered saline (containing 3.3 mM K^+) for 1.5 minutes and the supernatants containing released basal norepinephrine were collected for scintillation counting. The slices were then depolarized by exposure to buffered saline containing 30mM K^+ for 1.5 minutes and the supernatants, containing evoked norepinephrine, were also collected for scintillation counting. Slices were exposed to the desired concentration of peptide in all solutions from the time of loading with norepinephrine to the end of the experiment (about 2 hours). The data points are the differences of the means of 7 basal determinations and 7 evoked determinations at each drug concentration. Release in the absence of drug is taken as 100 per cent and the remaining points are scaled accordingly. The error bars are the standard errors of the means of the differences. Curves of best fit and the corresponding IC50's were derived. The single IC50

for SNX-111 is correlated with binding to site 1 calcium channels; the two IC50s for SNX-230 are for inhibition associated with binding to site 1 calcium channels (65nM) and to site 2 calcium channels (0.02 nM); the apparent single IC50 for SNX-183 is presumed to reflect binding to both site 1 and site 2 calcium channels with about equal affinity (see text). Evoked release in the absence of Ca^{++} in the buffer was equal to basal release (data not shown); thus all release shown is calcium-dependent release.

B. Inhibition of ATP release from Electric Organ Synaptosomes

Synaptosomes were prepared substantially as described in Example 3A. The diluted synaptosome suspension from the final centrifugation step was pelleted at 30,400 x g for 15 min and resuspended in 1 ml of synaptosome buffer (with the inclusion, for some experiments, of 1% BSA to enhance stability of the synaptosomes). This final synaptosome preparation was stored at 0°C and used for ATP release experiments within 30 hours. Storage for longer periods resulted in the almost complete loss of depolarization-dependent ATP release activity.

Luminometry was performed according to published method (Morel, Schweitzer). Into a 5 ml polypropylene test tube were mixed 465 μl synaptosome buffer, 5 μl of 5 μg/ml luciferin in PSB, 20 μl firefly lantern extract (1 Sigma FLE-50 bottle reconstituted in 1 ml PSB and spin-dialyzed through 3 ml of Sephadex G-25 pre-equilibrated in PSB), 5 μl 100 mM $CaCl_2$, and 5 μl synaptosome suspension (5-7 mg/ml protein, excluding BSA). The tube was placed in the chamber of a custom-built luminometer and the light output produced by extracellular ATP was continuously monitored by a chart recording of the voltage

generated by the photomultiplier tube. Exocytotic release of ATP was evoked by injecting 0.5 ml of high K+ buffer (synaptosome buffer with equimolar replacement of Na+ by K+) into the reaction mixture in the luminometer.

ATP release was quantitated by comparing the peak heights of unknowns with the heights of peaks generated by ATP standards that were injected into each reaction mixture at the end of each trial. Over the range investigated, light output was linear with respect to the amount of ATP injected. IC_{50} values were calculated from the dose-dependent ATP inhibition curves, and are reported in Table 6.

C. Inhibition of Dopamine release from rat striatal slices

Slices (0.3 x 0.3 x 1.5 mm) were prepared from rat striatum, and were pre-loaded with radiolabeled (tritiated) dopamine. Slices were perfused for 45 minutes in Krebs Ringer Bicarbonate buffer (oxygenated) as bathing medium. Release of neurotransmitter was stimulated by adding to the perfusion medium KCl at a concentration ranging between 4.8 and 15 mM, for a period of one minute. The first such exposure was termed S1. Perfusion with bathing medium was continued. Test compound(s) were introduced into the perfusion medium 20 minutes before the second stimulation (S2), which was done identically to S1. The ratio of S2/S1 was calculated to determine drug effects. A drug was considered to block release if S2/S1 was significantly less than unity.

D. Inhibition of Acetylcholine Release from Striatal Slices.

Release of acetylcholine was measured as described above in part C for dopamine release, except that slices were pre-loaded with radiolabelled choline instead of dopamine.

E. Inhibition of Electrically Stimulated Contractions of Guinea Pig Ileum

Guinea pigs (300-400 gms) were decapitated and the ileum removed. A section of ileum about 6 cm from the caecum was placed immediately into Krebb's modified buffer maintained at 37°C in a water bath, and aerated with a mixture of 95% O_2 and 5% CO_2. The buffer contains: KCl, 4.6 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; Glucose, 10.0 mM; NaCl 118.2 mM; $NaHCO_3$, 24.8 mM; $CaCl_2$, 2.5 mM.

Small pieces of ileum were cut and pulled over a glass pipette, scored and the logtudinal muscle removed. Each piece was attached to an electrode at one end and to a force transducer at the other end. The preparation was lowered into an organ bath maintained at 37°C and aerated with O_2:CO_2. The resting tension was set at 1 gm, and the tissue was stimulated at 30-50V with a duration of 4.5 msec per stimulation.

Baseline responses (contractions) were recorded for 10-15 min. and aliquots (100 ml) of drug were added to the bath until inhibition occurred. Following testing, tissues were washed until original response magnitude was achieved.

F. Inhibition of amino acid neurotransmitter release from rat brain slices

Male Sprague-Dawley rats were lightly anesthetized with ether, decapitated, and the brains removed to ice cold oxygenated basal medium (in mM: NaCl; 118, KCl, 4.8; $CaCl_2$, 1.3; $MgSO_4$, 1.2; KH_2PO_4,

1.2; glucose, 11). Hippocampus and cerebral cortex were further dissected from the brain and slices (300-400 μm thick) were prepared using a γ McIlwain Tissue Chopper at 4 degrees. Each slice was preincubated at 37 degrees for 15 minutes. Buffer was then replaced with an equal volume of either basal medium or stimulation medium (in mM: NaCl; 88, KCl, 30; $CaCl_2$, 1.3; $MgSO_4$, 1.2; KH_2PO_4, 1.2; glucose, 11). Incubation was then continued for 15 minutes. Tubes containing slices were then centrifuged for 1 minute in a Beckman Microfuge. The supernatants were collected and heated for 10 minutes at 100 degrees. Aliquots (20ul) were used for analysis of amino acid content using pre-column derivatization with o-phthalaldehyde followed by HPLC as described by Newcomb.

Figure 13B shows the effect of MVIIA OCT on K-stimulated release of amino acid neurotransmitters (Aspartate, GABA, glutamate) contrasted to effects on serine, which is not a neurotransmitter. Significant reductions in the amount of GABA and glutamate were observed, as reported in Table 7.

Example 7

Rat Tail-Flick Assay for Analgesia

Male Sprague-Dawley rats (250-300g; Simonsen) were implanted with intrathecal (i.t.) catheters, which were inserted through the atlanto-occipital membrane and threaded subdurally about 8 cm therefrom. Animals were not used in experiments until at least 2 days following implantation.

To perform the Tail-FLick test, a rat was restrained in a plastic cone having openings at each end, and was placed on a platform, positioned such

that its tail hung down from the platform in close proximity to a heating bulb. Latency to flick the tail away from the bulb was recorded. A trial consisted of four such flicks at 1-2 min. intervals, where the first latency time was generally not used, and the three subsequent tests were averaged. Latencies measured in the absence of analgesic agent(s) were recorded for each rat as "Baseline latency."

Rats were then removed from the restraining cones, and injected (i.t.) with test compound in a volume of 5 μl, followed by 10 μl saline. Animals were subjected to post-drug trials at one or more time intervals thereafter (usually 25 min and 45 min.), as described above. In the cases where drug enhancement was tested, test compound was first injected, followed by tail-flick trials, to assess the potency of the drug alone. Approximately 1 hour later, a known analgesic, such as morphine, was injected, and trials repeated.

Drug effects were calculated as follows:

$$\% \text{ Effect} = 100 \times \frac{(\text{post-drug latency}) - (\text{baseline latency})}{(\text{maximum latency}) - (\text{baseline latency})},$$

where maximum latency was measured as experimental cut-off time, the time beyond which the tail was not allowed by the experimenter to be exposed to heat, due to risk of burn to the animal.

Example 8

Rat Formalin Test for Analgesia

Rats (male Sprague-Dawley, 275-300 g) were implanted with lumbar intrathecal catheters under halothane anesthesia. Catheters extended from the cisterna to the rostral edge of the lumbar enlargement. 3-5 days after implant, animals without

motor dysfunction were used.

Animals were examined for the effects of drugs given in the formalin test, in which 50 ul of 5% formalin was injected on the plantar surface of the paw. The number of flexions of the paw were counted at intervals after the injection of the formalin. Drugs tested in this assay were dissolved in sterile saline (0.9% NaCl) and injected in a volume of 10 ul followed by 10 ul to clear the catheter.

Injection of formalin alone or with vehicle (saline) resulted in a biphasic response pattern of hind paw withdrawals (see Figure 16). The area under the curve of the flinches/min was calculated for phase 1 (time = 0-10 min) and phase 2 (10-60 min). These values were plotted versus the intrathecal log dose (ug) and the results are shown in Figure 17.

Example 9

Rat model of peripheral neuropathy

Male Sprague-Dawley rats (200-350 gm) were prepared with chronic lumbar intrathecal catheters inserted under halothane anesthesia (Yaksh and Rudy). Animals were placed in a prone position and the left paraspinal muscles were separated from the spinous processes at the L_4-S_2 levels, as described by Kim et al. The left L5 and L6 nerve roots were exposed and tightly ligated with 6-0 surgical silk suture. The rats were allowed to recover from anesthesia. Allodynia was typically observed to occur beginning 1-2 days post-surgery and continuing for as long as 45 days.

For testing, animals were placed in plastic cubicles with open wire mesh bottoms. Compound dissolved in preservative-free saline solution was administered in a volume of 10 μl through the

intrathecal catheter, followed by 10 μl saline to flush the catheter line. Animals were tested for allodynia at various time points after drug treatment, as described below.

To assess the threshold of a non-noxious stimulus required to produce a left hind paw withdrawal (allodynia), Von Frey hairs (ranging from 0.4-15 grams), were systematically applied to the surgically treated hind paw. Failure to evoke a response was cause to test the next stiffer hair. Evocation of a brisk withdrawal response was cause to test the next lower stimulus intensity. This paradigm was repeated according to a statistical method (Dixon) to define the 50% response threshold. Allodynia was evidenced by a threshold less than 3 grams (referring to the hair stimulus intensity) exhibited by all surgically treated animals.

Results of animals treated with saline, 0.3, 1, or 3 μg of SNX-111 are shown in Figures 18 and 19. Data in Figure 17 are expressed as percent maximum effect, where the maximum effect indicates a complete reversal of allodynia, or insensitivity to stimulus (maximum equals 15 gram hair cutoff). A baseline of zero indicates a mean sensitivity less than 3 grams. As shown in Figure 18, treatment of rats (n=6/treatment) with 1 or 3 μg SNX-111 resulted in elevation of threshold response. Peak effects were observed by 30-60 minutes, and effects lasted in excess of 60 minutes.

The results shown in Figure 18 were summarized and subjected to statistical analyses (1-way ANOVA; Games-Howell test) in Figure 19. Also shown in Figure 18 is the response of animals treated with 10 μg morphine sulfate. In the study shown, treatment of rats with 3 μg SNX-111 or 10 μg morphine resulted

in significant reversal of allodynia in comparison to saline treated animals.

Animals were also observed for the appearance of general motor dysfunction, as evidenced by inability to ambulate symmetrically and for any other overt signs of unusual activity, such as tremor. No effects on motor activity were observed in saline or morphine-treated animals; a dose-dependent tremor characteristic of SNX-111 administration was observed in animals given SNX-111.

Although the invention has been described with respect to particular embodiments, it will be apparent to those skilled that various changes and modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A method of producing analgesia in a mammalian subject, comprising

administering to the subject, an omega conopeptide selected from the group consisting of TVIA (SNX-185), MVIIA (SNX-111) and derivatives thereof which are effective (a) to inhibit voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the peptide's ability to inhibit electrically stimulated contraction of the guinea pig ileum, and (b) to bind to omega conopeptide MVIIA binding sites present in neuronal tissue.

2. The method of claim 1, wherein the activities of the omega conopeptide in calcium-channel inhibition and in binding to the MVIIA binding site are within the ranges of such activities of omega-conotoxins MVIIA and TVIA.

3. The method of claim 1, wherein the omega conopeptide is MVIIA or TVIA.

4. The method of claim 1, wherein the omega conopeptide is SEQ ID NO: 30 (SNX-236).

5. The method of claim 1, wherein the analgesia is produced in a subject exhibiting neuropathic pain.

6. The method of claim 1, wherein the omega conopeptide is administered intrathecally.

7. A method of enhancing the analgesic effect produced by administration of opiates to a mammalian

	1 5 10 15 20 25 30
MVIIA/ SNX-111	C K G G K G A K C S R L M Y D C C T G S C - R - S G K - C
MVIIB/ SNX-159	C K G G K G A S C H R T S Y D C C T G S C N R - - G K - C
GVIA/ SNX-124	C K S X G S S C S X T S Y N C C R - S C N X Y T - K R C - - Y
GVIIB/ SNX-178	C K S X G T X C S R G M R D C C T - S C L L Y S N K - C R R Y
RVIA/ SNX-182	C K P X G S X C R V S S Y N C C S - S C K S Y - N K K C G

Fig. 1

TVIA/ SNX-185	C L S X G S S C S X T S Y N C C R - S C N X Y S - R K C R
SVIB/ SNX-183	C K L K G Q S C R K T S Y D C C S G S C G R - S G K - C
MVIIC/ SNX-230	C K G K G A P C R K T M Y D C C S G S C G R - R G K - C
SNX-231	C K G K G A X C R K T M Y D C C S G S C G R - R G K - C

Fig. 1 (con't)

MVIIA C K G K G A K C S R L M Y D C C T G S C R S G K C -NH_2
(SNX-111)

SNX-190	- - - A - - - - - - - - - - - - - - - - - -	NH_2
SNX-191	- A -	NH_2
SNX-193	- -	G-OH
SNX-194	- - - - - - - - - - -Nle- - - - - - - - - -	NH_2
SNX-195	- A -	NH_2
SNX-196	N- -	G-OH
SNX-197	NS- - - - - - - - - - - - - - - - - - - -	NH_2
SNX-198	- - - - - - - - - - - - - - - - - - A - - -	NH_2
SNX-200	- - - - - - - A - - - - - - - - - - - - - -	NH_2
SNX-201	- - - - - - - - - R K T S - - - - - - - - -	NH_2

Fig. 2

SNX-207 - - - - -

SNX-236 - - - - - - - - - - R L M - - - - - - - - - P - - - - - - NH_2

Fig. 2 (con't)

Fig. 3

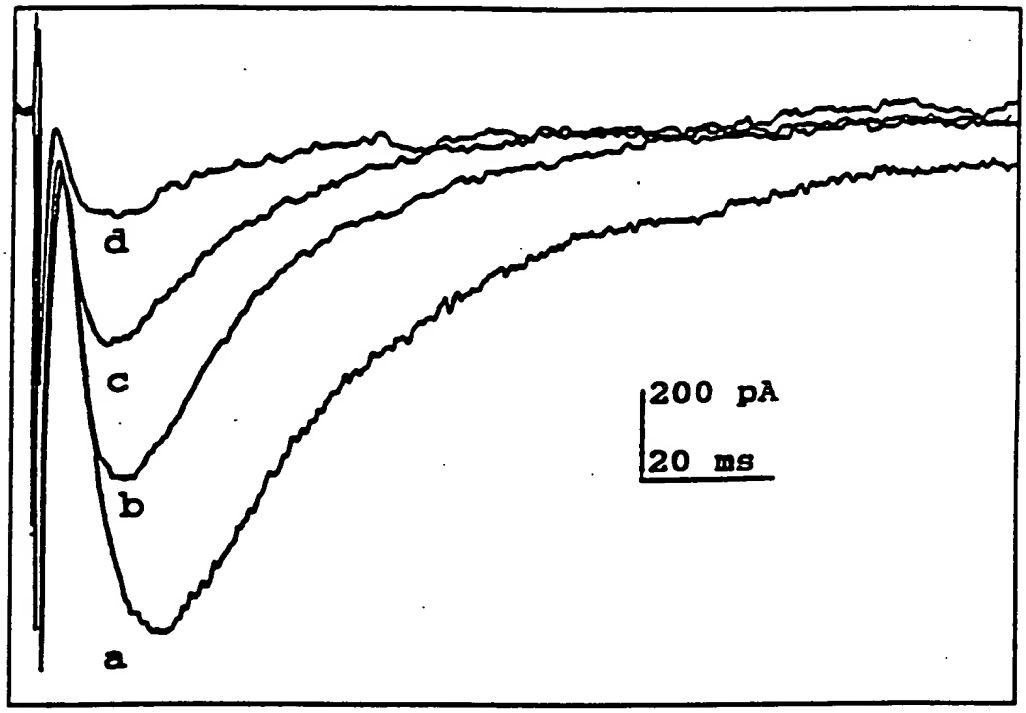

d
c
b
a
200 pA
20 ms

Fig. 4

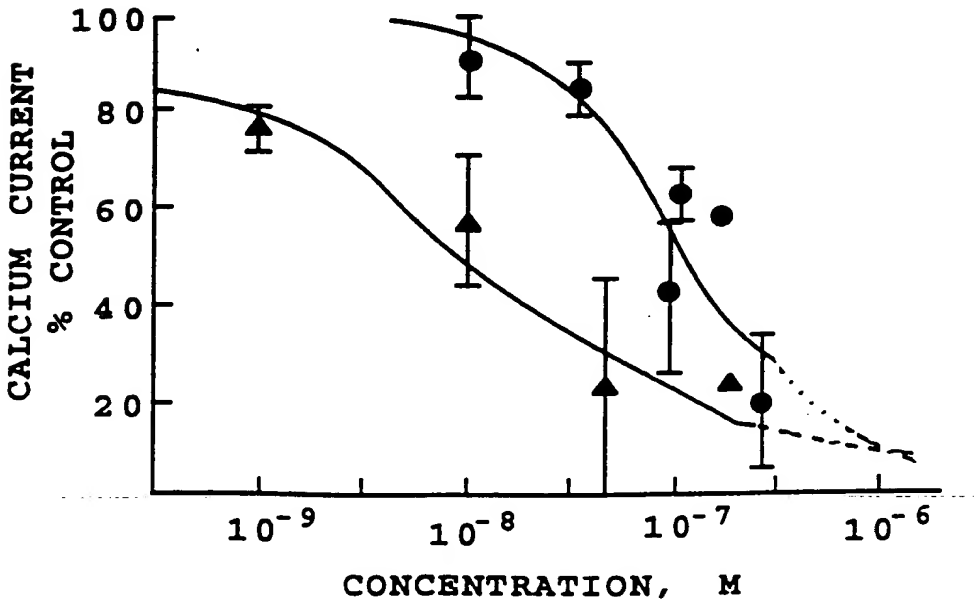
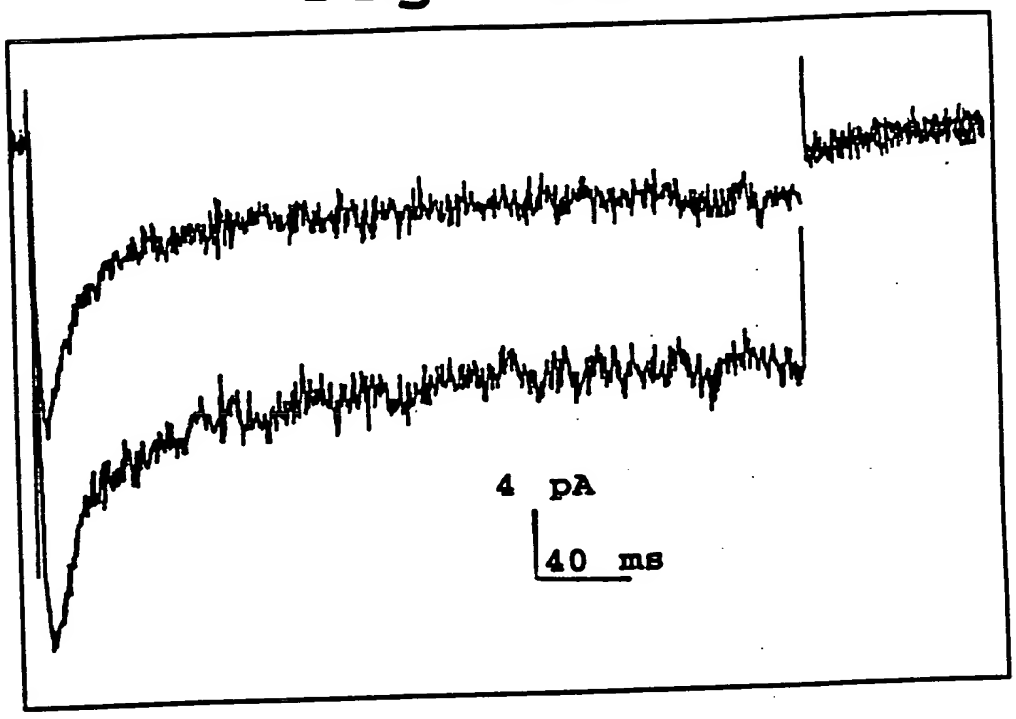

100
80
60
40
20
CALCIUM CURRENT
% CONTROL
10-9
10-8
10-7
10-6
CONCENTRATION, M

Fig. 5A


4 pA
40 ms

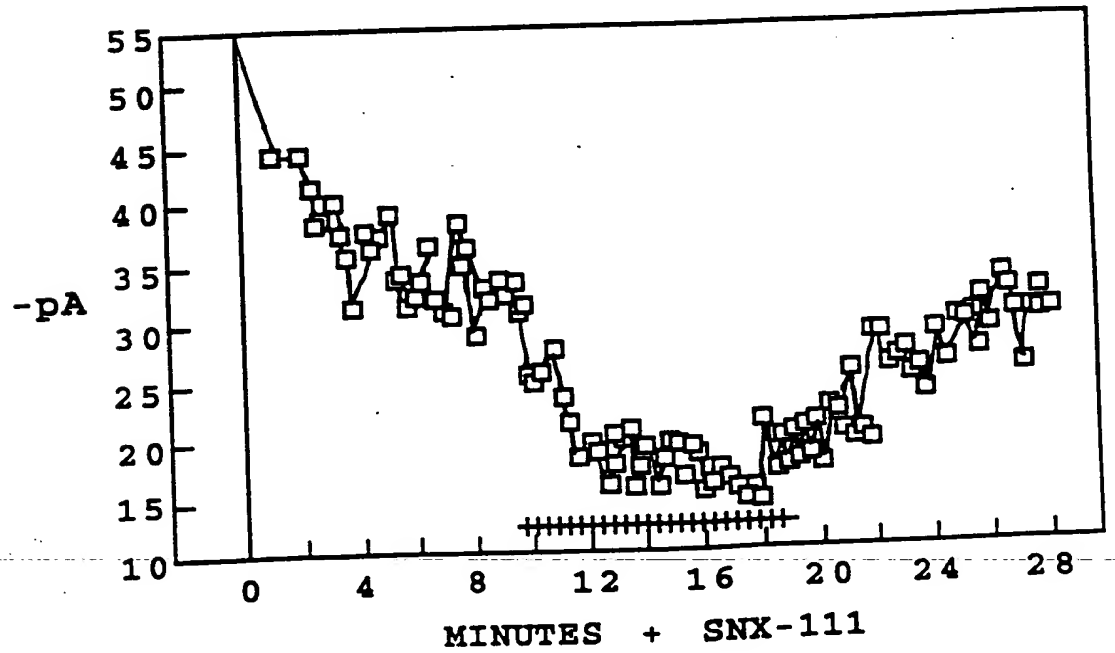

-pA
55
50
45
40
35
30
25
20
15
10
0
4
8
12
16
20
24
28
MINUTES + SNX-111

Fig. 5B

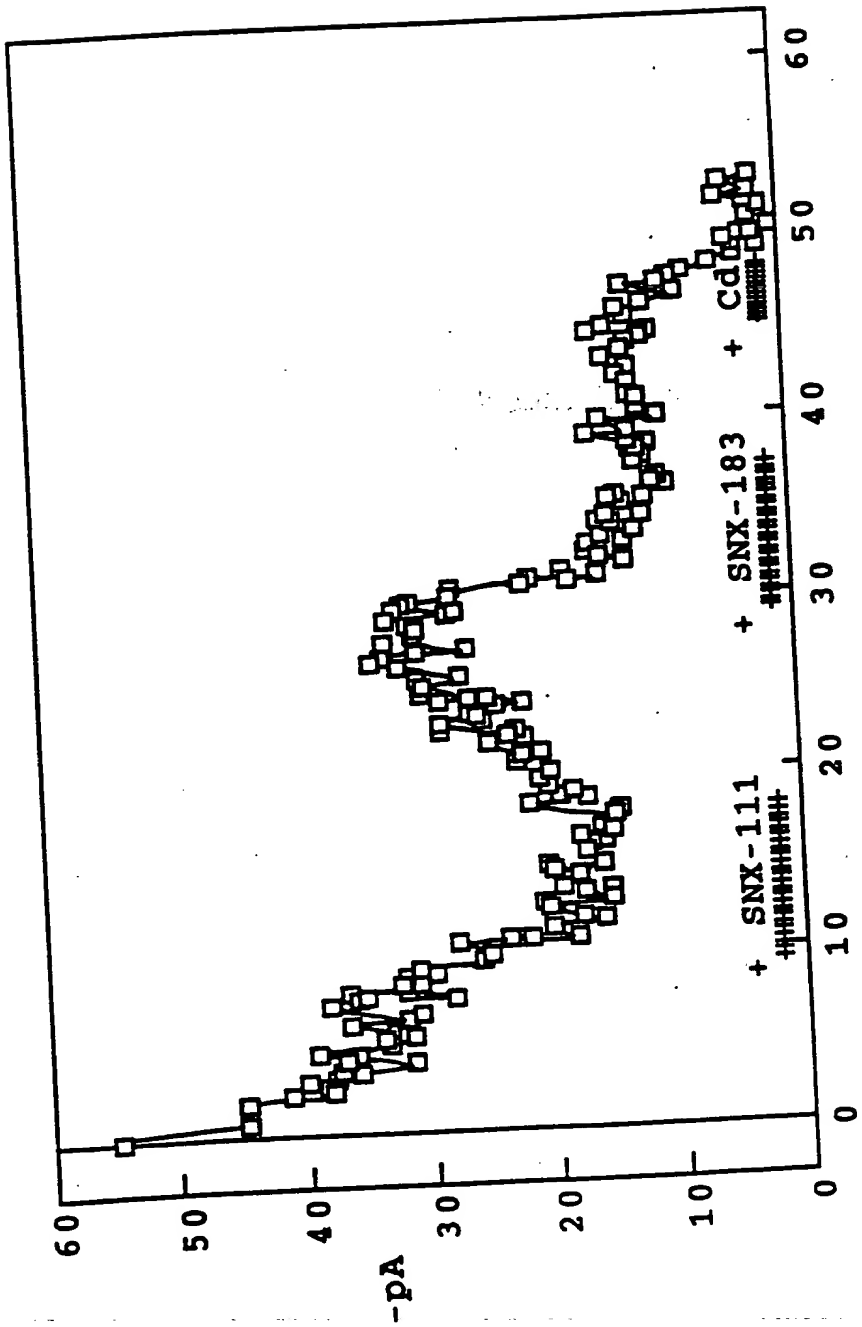
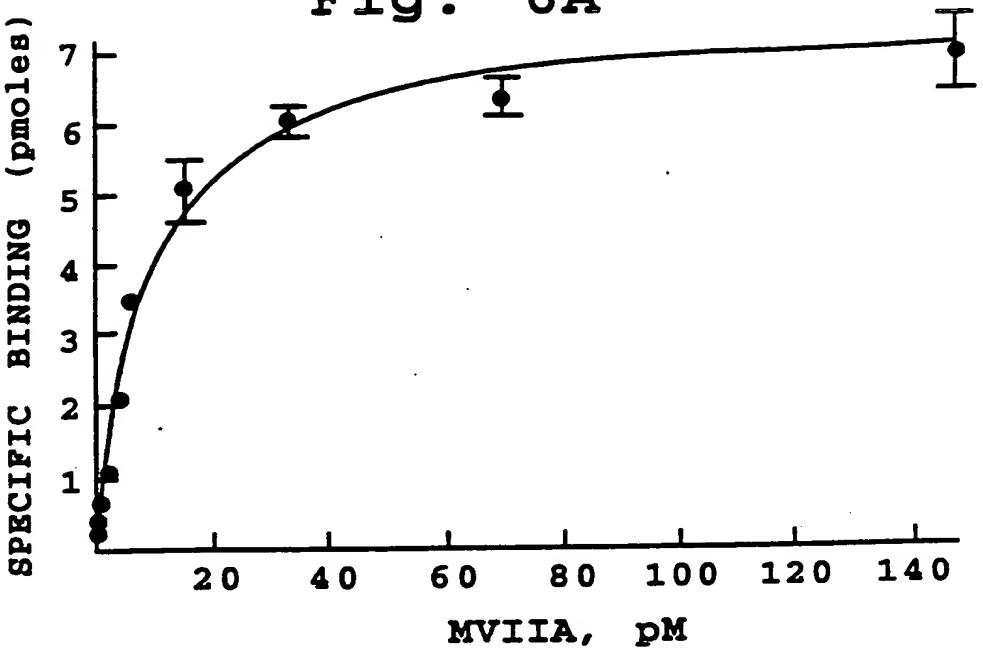


Fig. 5C

Fig. 6A


SPECIFIC BINDING (pmoles)
1
2
3
4
5
6
7
20
40
60
80
100
120
140
MVIIA, pM

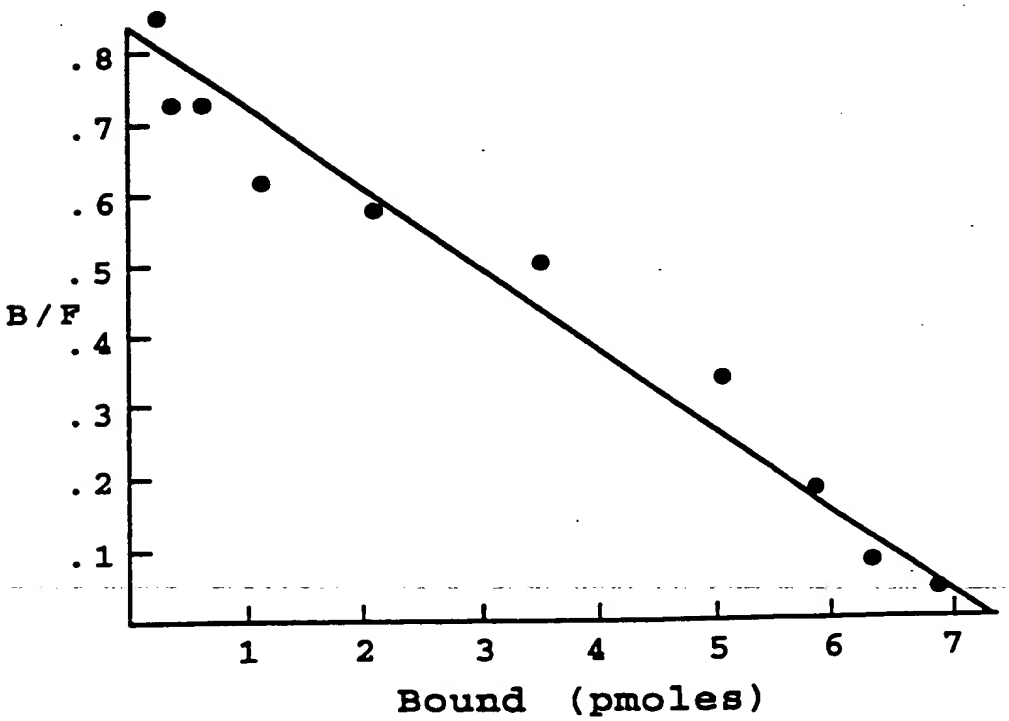

B/F
.1
.2
.3
.4
.5
.6
.7
.8
1
2
3
4
5
6
7
Bound (pmoles)

Fig. 6B

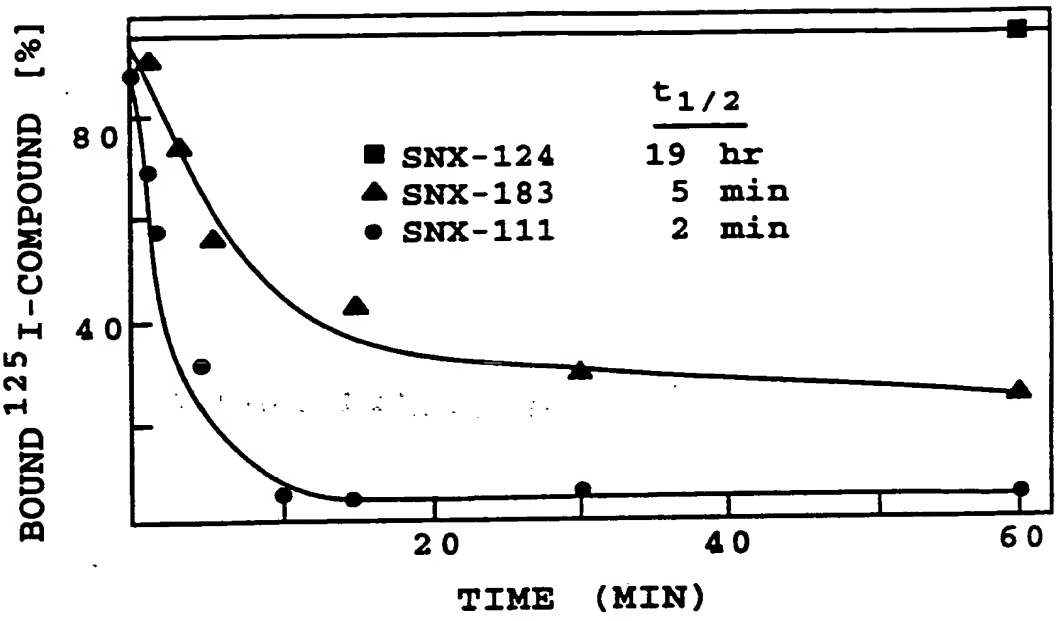

BOUND 125I-COMPOUND [%]
80
40
20
40
60
TIME (MIN)
t1/2
SNX-124 19 hr
SNX-183 5 min
SNX-111 2 min

Fig. 7

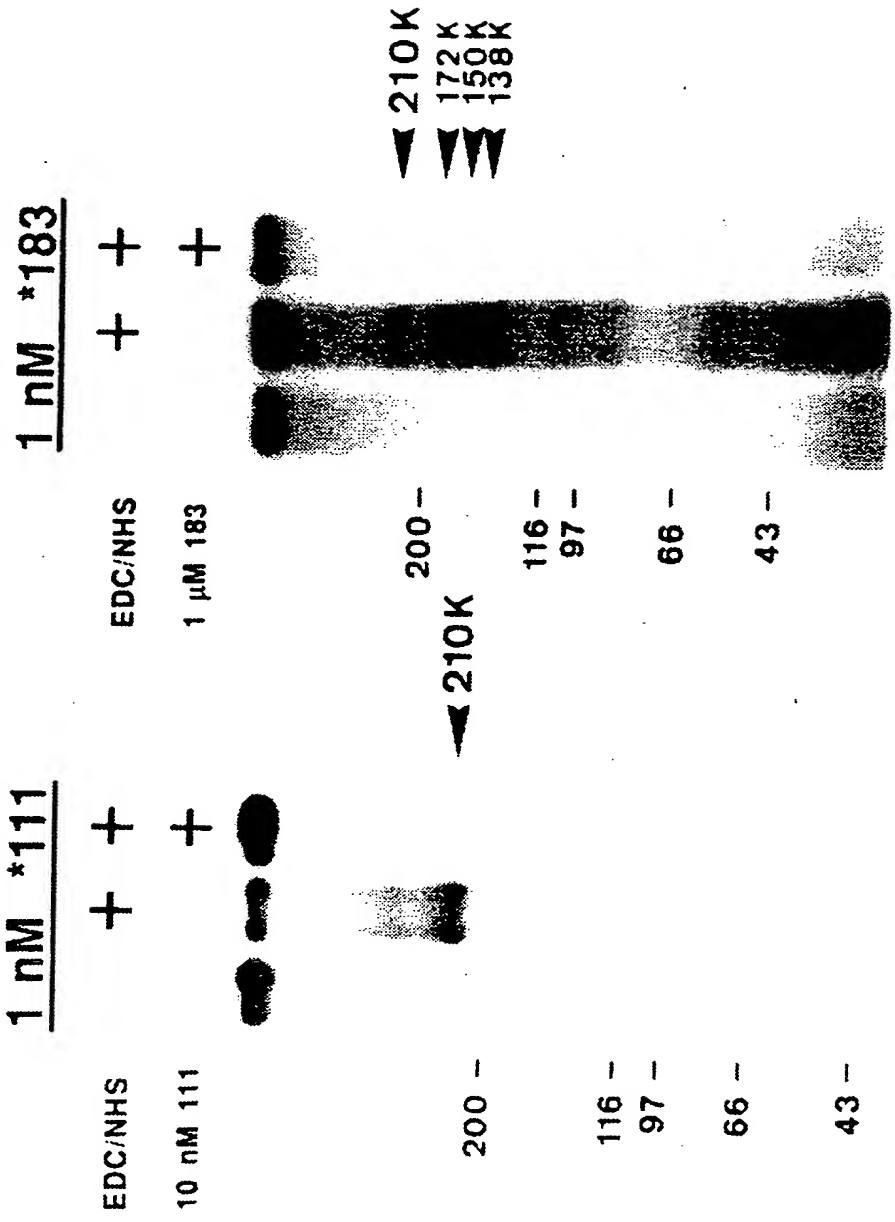


Fig. 10A

Fig. 10B

Fig. 8A

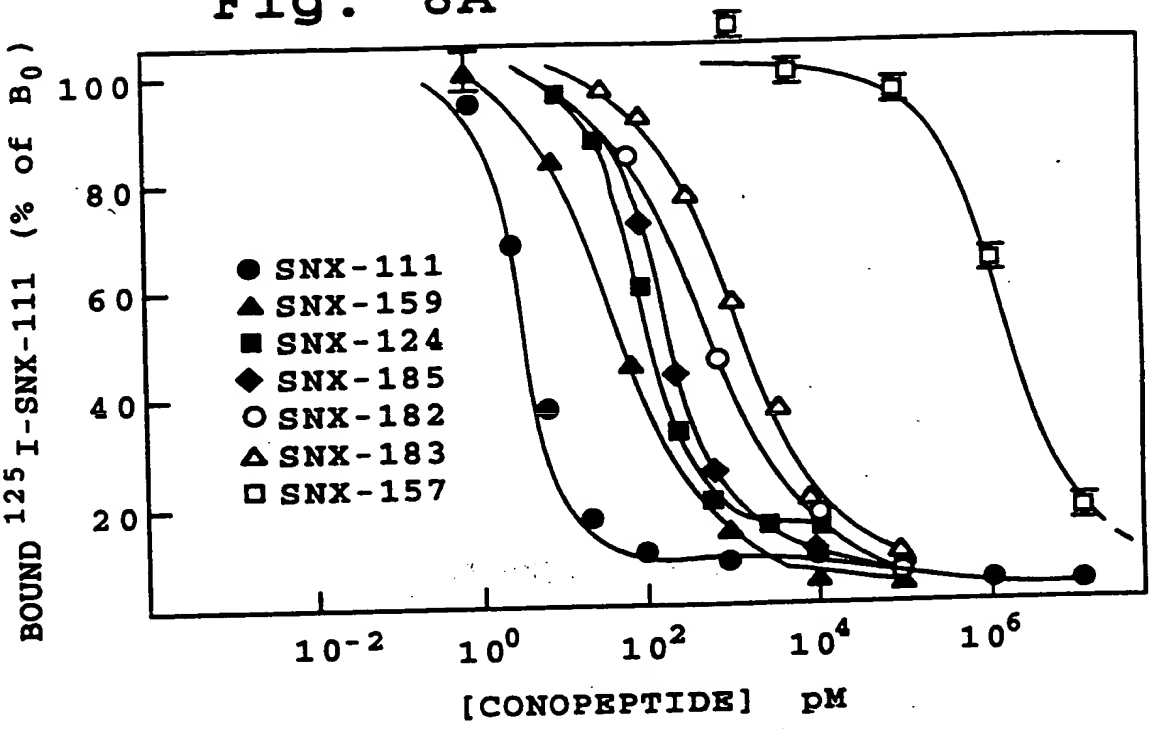

BOUND ^{125}I-SNX-111 (% of B_0)
100
80
60
40
20
SNX-111
SNX-159
SNX-124
SNX-185
SNX-182
SNX-183
SNX-157
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
[CONOPEPTIDE] pM

Fig. 8B

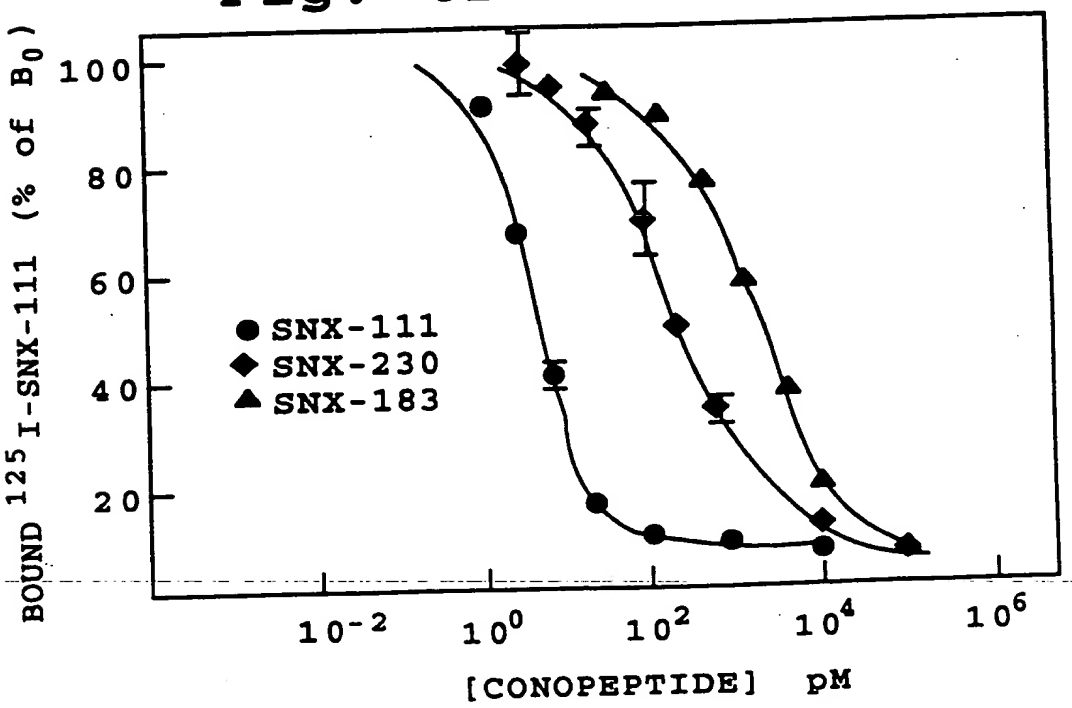

BOUND ^{125}I-SNX-111 (% of B_0)
100
80
60
40
20
SNX-111
SNX-230
SNX-183
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
[CONOPEPTIDE] pM

Fig. 9A

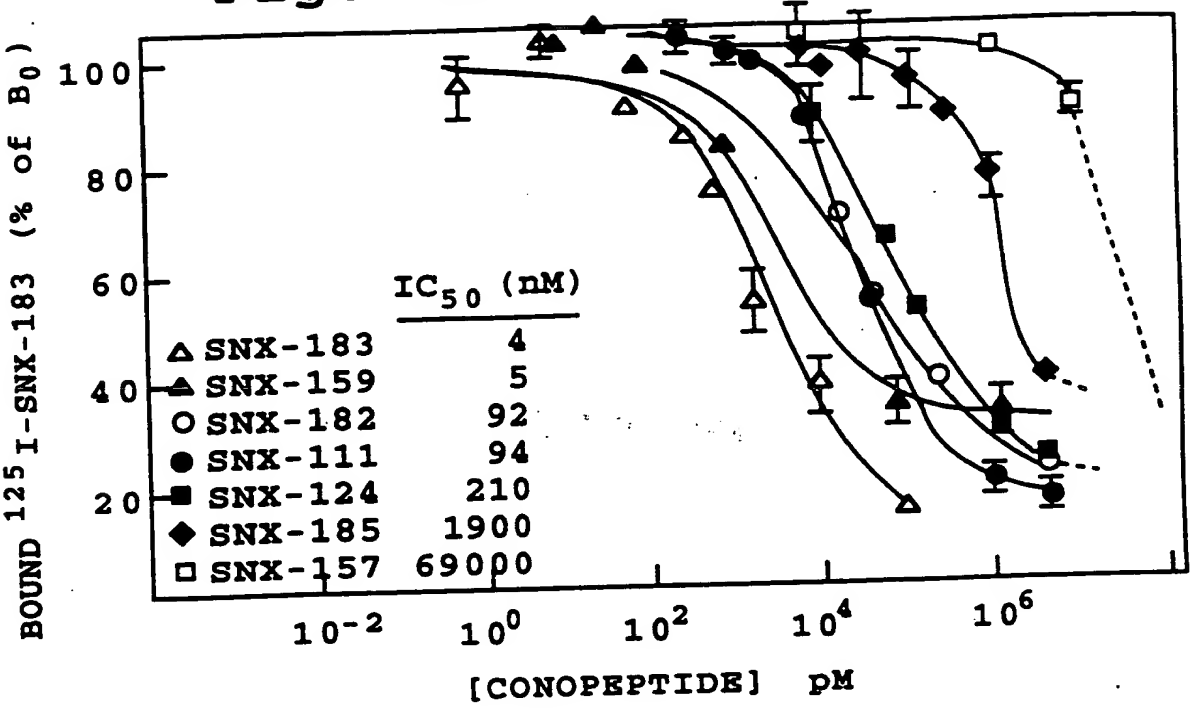

BOUND ^{125}I-SNX-183 (% of B_0)
100
80
60
40
20
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
[CONOPEPTIDE] pM
IC_{50} (nM)
SNX-183 4
SNX-159 5
SNX-182 92
SNX-111 94
SNX-124 210
SNX-185 1900
SNX-157 69000

Fig. 9B

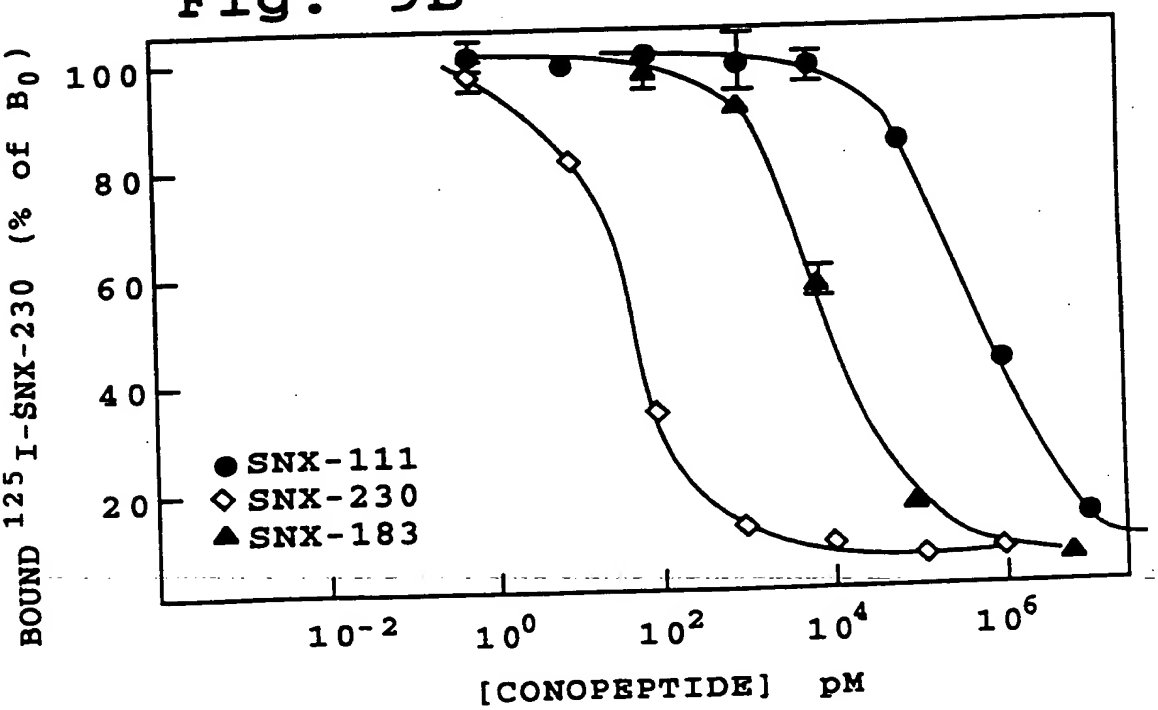

BOUND ^{125}I-SNX-230 (% of B_0)
100
80
60
40
20
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
[CONOPEPTIDE] pM
SNX-111
SNX-230
SNX-183

Fig. 11A

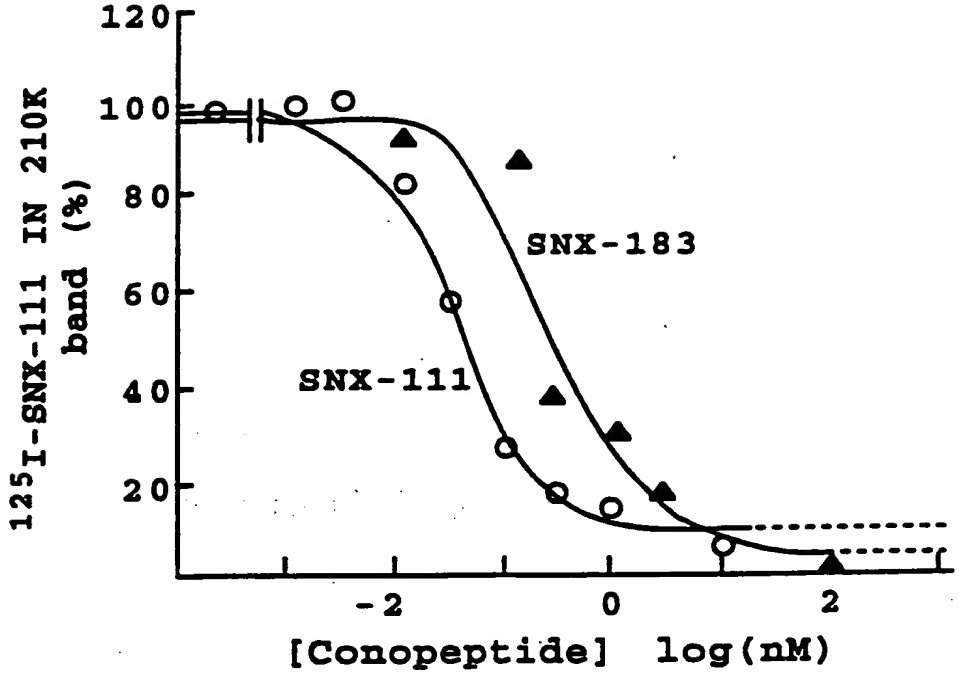
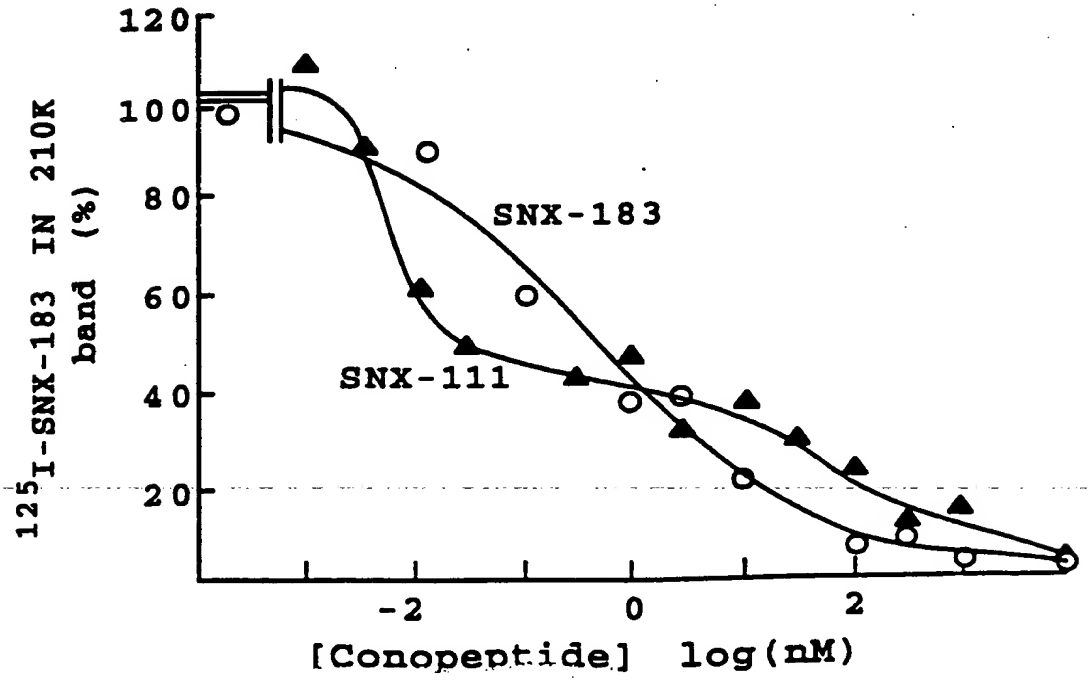


Fig. 11B



ROSTRAL SECTIONS

*111

Fig. 12A

CAUDAL SECTIONS

Fig. 12B

*111
+
excess
111

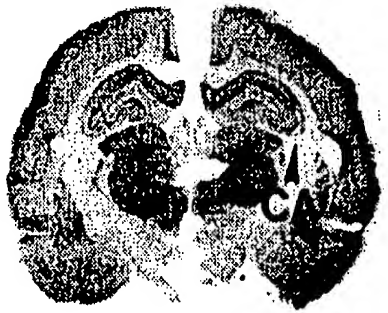
Fig. 12C

Fig. 12D

ROSTRAL SECTIONS

CAUDAL SECTIONS

*183



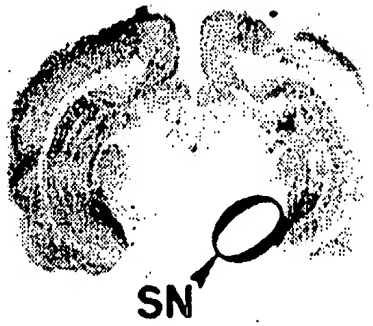


Fig. 12E

Fig. 12F

*183
+
excess
183

Fig. 12G

Fig. 12H

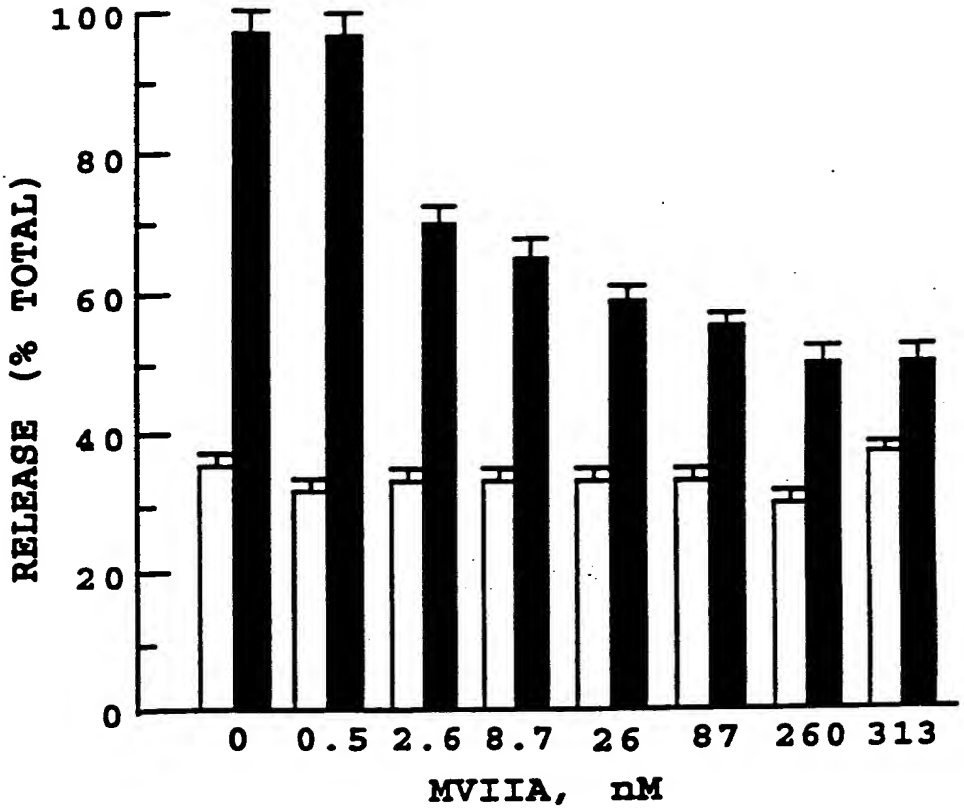


Fig. 13A

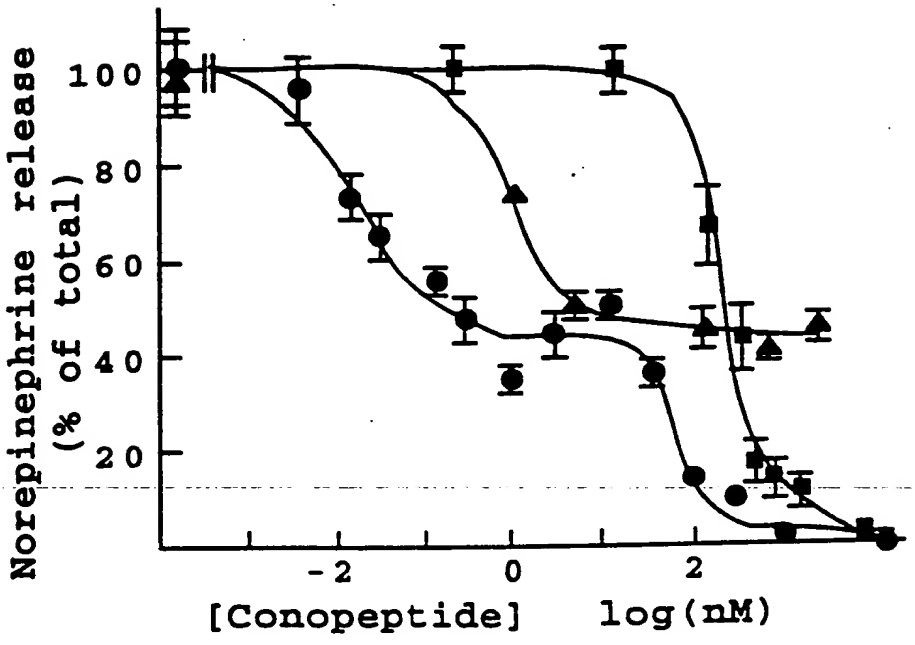


Fig. 13B

```
          1         5         10        15        20        25        30
I.
MVIIA     C K G K G A K C S R L M Y D C C T G S C - R - S G K - C
MVIIB     C K G K G A S C H R T S Y D C C T G S C N R - - G K - C

II.
TVIA      C L S X G S S C S X T S Y N C C R - S C N X Y S R K - C R
SNX-      C L S X G S S C S R L M Y N C C R - S C N X Y S R K - C R
207
SNX-      C L S X G S S C S R L M Y N C C R - S C N P Y S R K - C R
236

III.
RVIA      C K P X G S X C R V S S Y N C C S - S C K S Y - N K K C G
SVIA      C R S S G S X C G V T S I - C C - G R C - - Y R G K - C T
GVIIA     C K S X G T X C S R G M R D C C T - S C L L Y S N K - C R R Y D
SVIB      C K L K G Q S C R K T S Y D C C S G S C G R - S G K - C
MVIIC     C K G K G A P C R K T M Y D C C S G S C G R - R G K - C
SNX-      C K G K G A X C R K T M Y D C C S G S C G R - R G K - C
231
```

Fig. 14

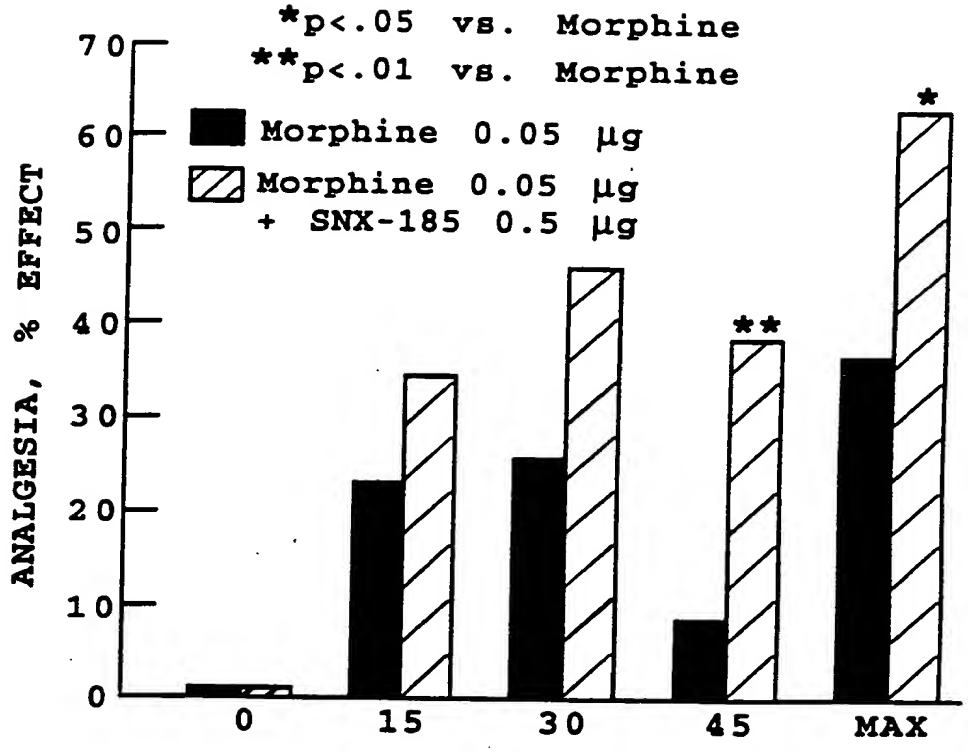


Fig. 15

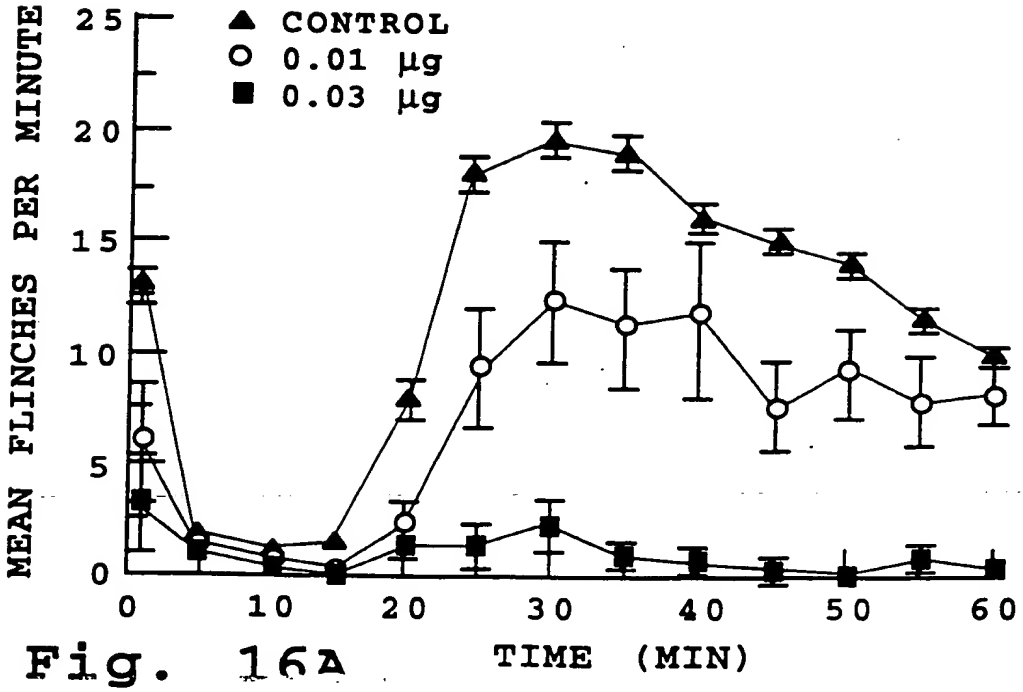


Fig. 16A

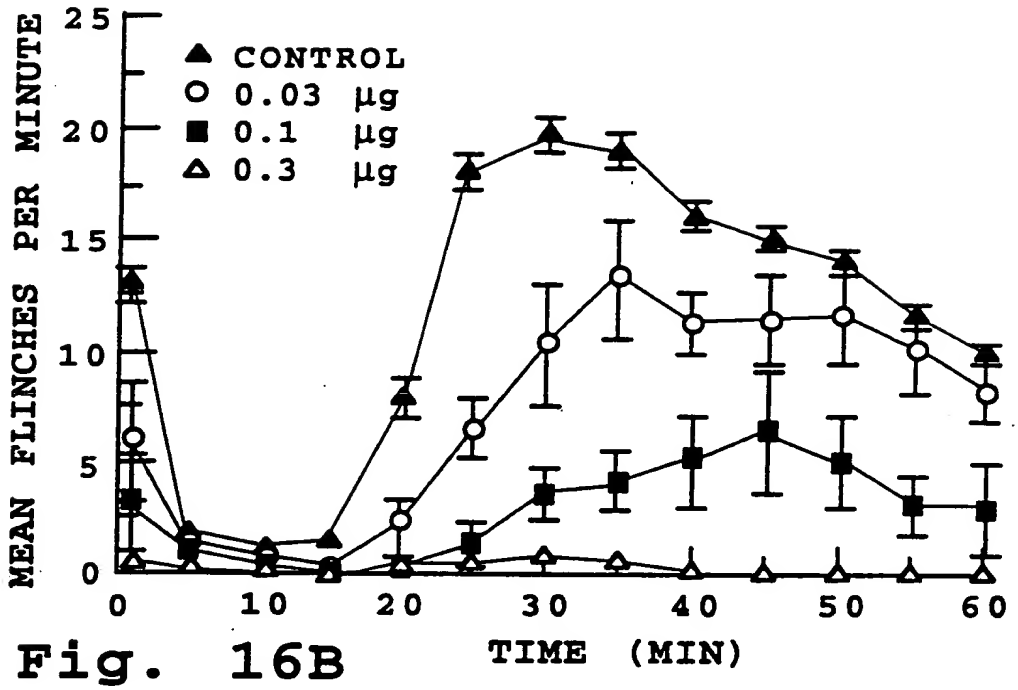


Fig. 16B

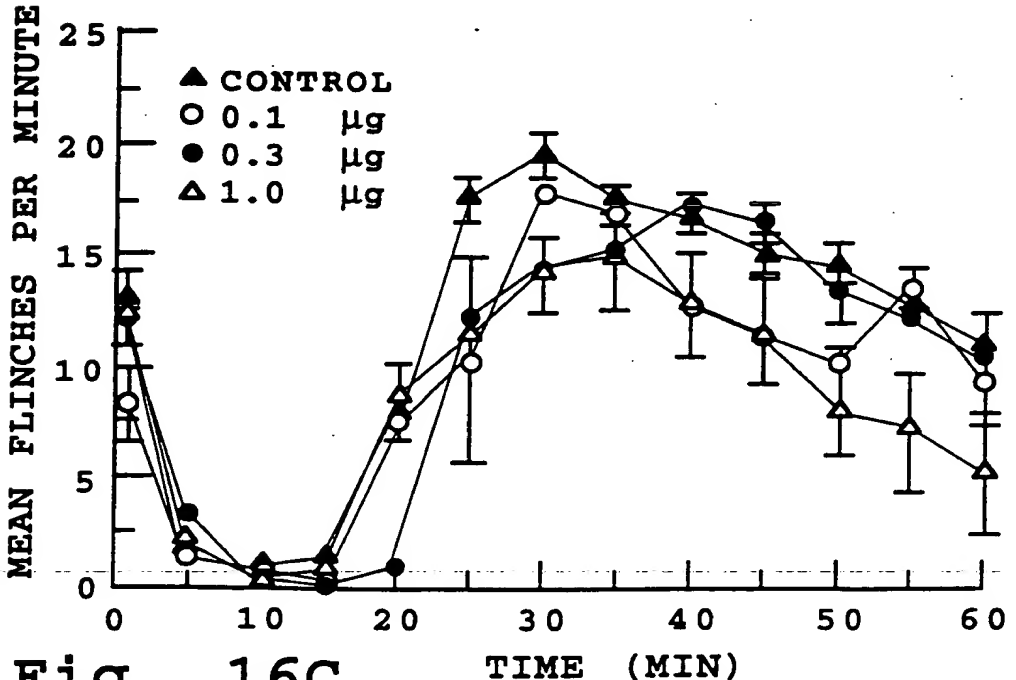


Fig. 16C

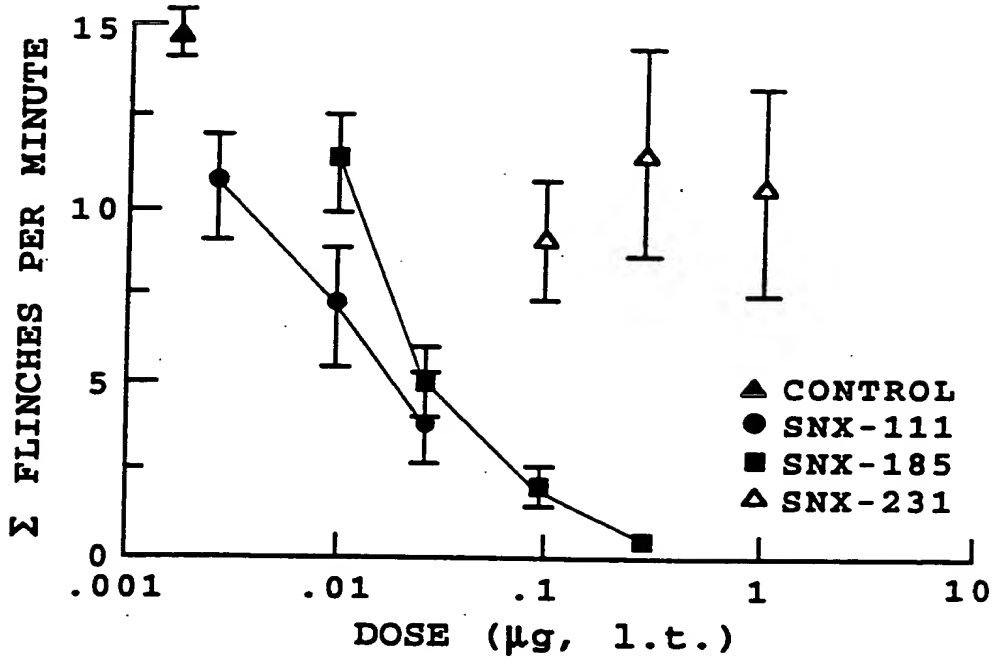


Fig. 17A

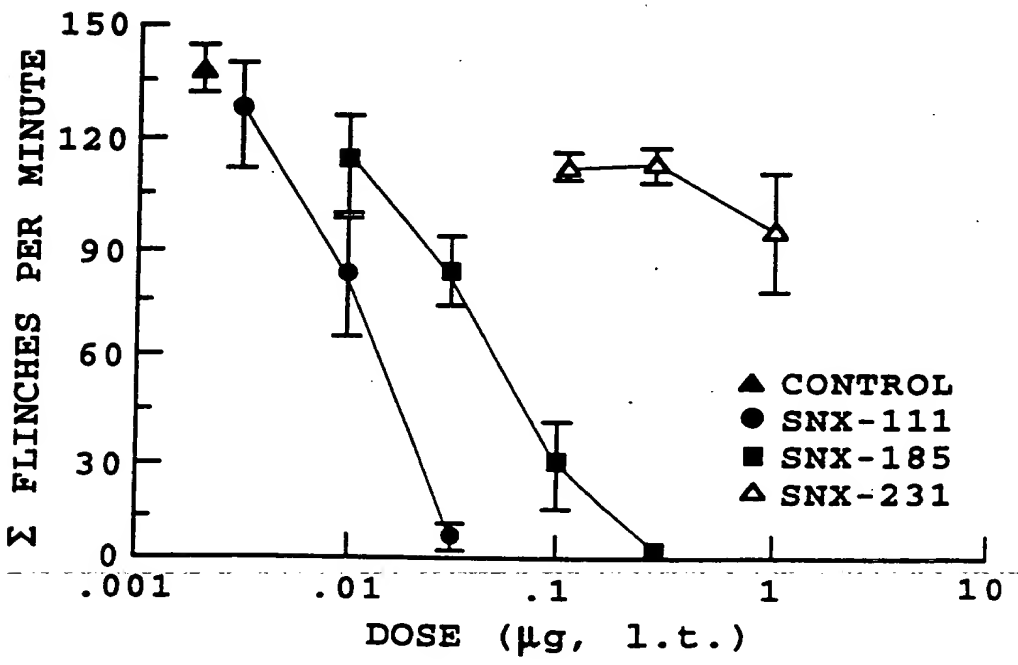


Fig. 17B

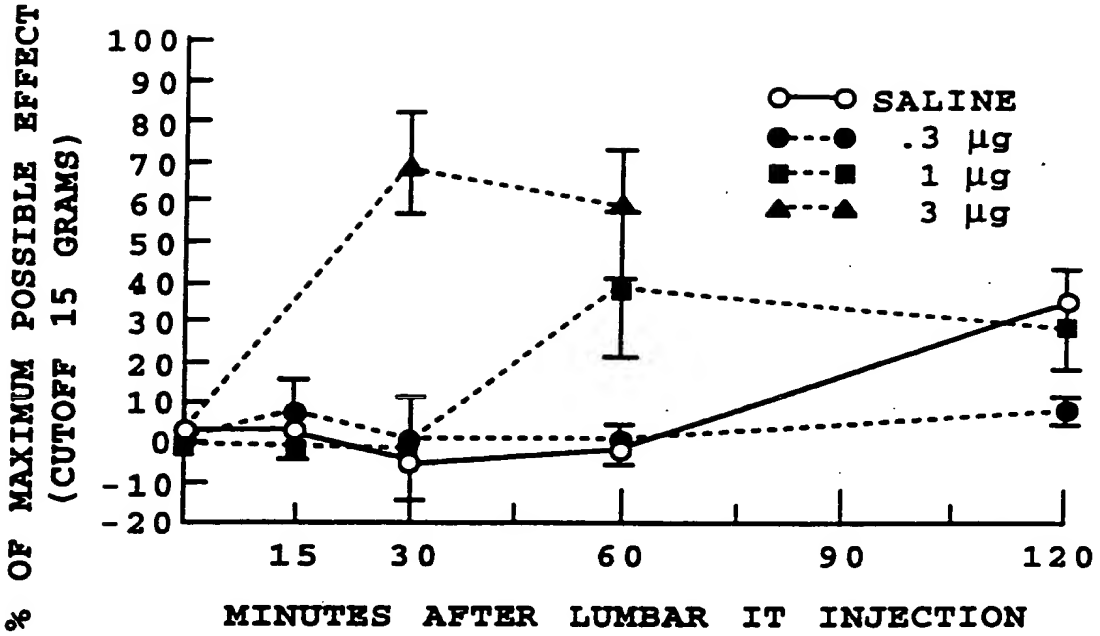

% OF MAXIMUM POSSIBLE EFFECT
(CUTOFF 15 GRAMS)
100
90
80
70
60
50
40
30
20
10
0
-10
-20
SALINE
.3 µg
1 µg
3 µg
15
30
60
90
120
MINUTES AFTER LUMBAR IT INJECTION

Fig. 18

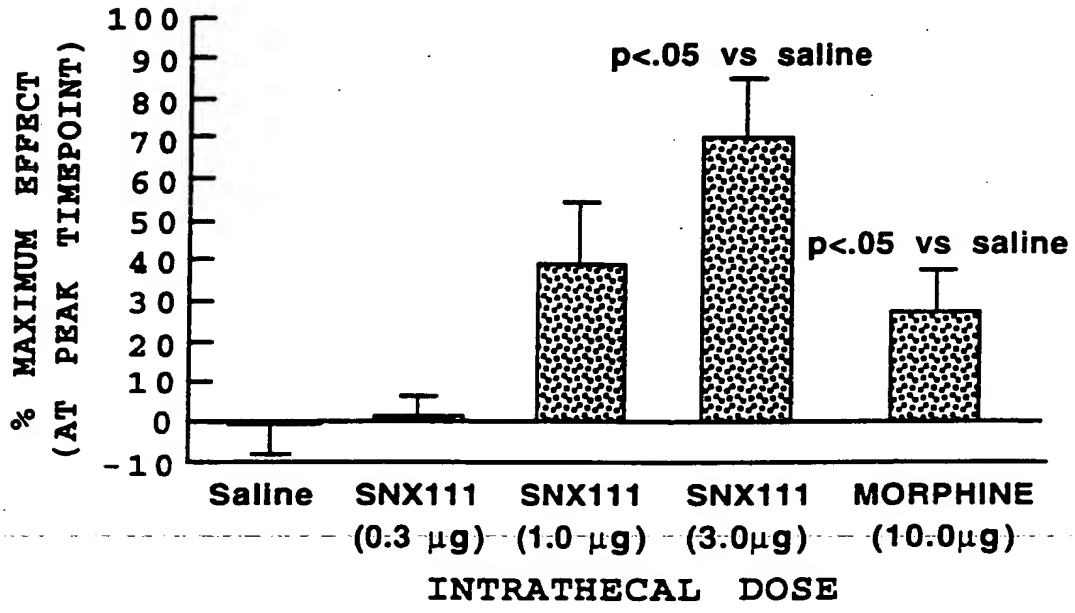

% MAXIMUM EFFECT
(AT PEAK TIMEPOINT)
100
90
80
70
60
50
40
30
20
10
0
-10
p<.05 vs saline
p<.05 vs saline
Saline
SNX111 (0.3 µg)
SNX111 (1.0 µg)
SNX111 (3.0µg)
MORPHINE (10.0µg)
INTRATHECAL DOSE

Fig. 19

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/11349

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/10; A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EUROPEAN JOURNAL OF PHARMACOLOGY vol. 218, no. 1, 21 July 1992, pages 75 - 81 L. BASILICO ET AL. 'Influence of omega-conotoxin on morphine analgesia and withdrawal syndrome in rats' see results on pages 77-78 see discussion on pages 78-79 ---	1,2,5-8
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 89, no. 9, 1 May 1992, WASHINGTON US pages 3825 - 3829 K. MACKIE AND B. HILLE 'Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells' see page 3827, right column, paragraph 2 - page 3828, left column, paragraph 1; figure 4 --- -/--	7,8

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 APRIL 1993	27.04.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	FUHR C.K.B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	NEUROSCIENCE LETTERS vol. 126, no. 1, 13 May 1991, pages 67 - 70 M. OCANA AND J.M. BAEYENS 'Analgesic effects of centrally administered aminoglycoside antibiotics in mice' see page 67, left column, paragraph 1 - right column, paragraph 1 see page 69, left column, paragraph 5 - right column, paragraph 2	1,5,6
A	PAIN vol. 41, no. 3, June 1990, AMSTERDAM, NETHERLANDS pages 365 - 371 W.A. PRADO ET AL. 'Antinociception induced by intraperitoneal injection of gentamicin in rats and mice' see page 369, right column, paragraph 3	1,5-7
A	WO,A,9 107 980 (NEUREX CORPORATION) 13 June 1991 see page 11, line 5 - page 15, line 25; claims 1-13; figures 1,2,14	1-3,11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/11349

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-10 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort jusufying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9211349
SA 68886

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 14/04/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9107980	13-06-91	US-A-	5051403	24-09-91
		AU-A-	6964091	26-06-91
		US-A-	5189020	23-02-93